

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 06 September 2000 (06.09.00)	
<b>International application No.</b> PCT/GB00/00219	<b>Applicant's or agent's file reference</b> WPP81798
<b>International filing date (day/month/year)</b> 24 January 2000 (24.01.00)	<b>Priority date (day/month/year)</b> 22 January 1999 (22.01.99)
<b>Applicant</b> MORGAN, James, Alun, Wynne et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

16 August 2000 (16.08.00)

☐ in a notice effecting later election filed with the International Bureau on:
2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<b>The International Bureau of WIPO</b> 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	<b>Authorized officer</b> Juan Cruz Telephone No.: (41-22) 338.83.38
--	--

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>WPP81798</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/GB 00/ 00219</b>	International filing date (day/month/year) <b>24/01/2000</b>	(Earliest) Priority Date (day/month/year) <b>22/01/1999</b>
Applicant <b>Horticulture Research International et al</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

### 1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☒ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☒ Certain claims were found unsearchable (See Box I).

3. ☐ Unity of invention is lacking (see Box II).

4. With regard to the title,

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

**BIOLOGICAL CONTROL OF NEMATODES**

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

☒ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

1

☐ None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 00/00219

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No

GB 00/00219

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A01N63/00 A01N63/02 C12N15/31 C12P21/00 C07K14/24  
 //(C12P21/00,C12R1:01)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>WO 99 22598 A (UNIV READING ;ELAWAD SAMI            ABDULRAHAMAN (GB); HAGUE NIGEL GRAHAM MA)            14 May 1999 (1999-05-14)            cited in the application            page 1 -page 2            page 4, line 7 - line 14            page 5, line 2 - line 8            page 5, line 25 - line 28            page 8, line 1 - line 15; claims            1-3,9,10,14; example 5</p> <p style="text-align: center;">--- -/-</p>	1-9, 11, 29



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 May 2000

Date of mailing of the international search report

13/06/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3018

Authorized officer

Muellners, W

## INTERNATIONAL SEARCH REPORT

International Application No

GB 00/00219

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	<p>DATABASE BIOSIS 'Online!  BIOSCIENCES INFORMATION SERVICE,  PHILADELPHIA, PA, US  AN 2000:109497,  GREWAL, PARWINDER S. (1) ET AL:  "Allelopathy: A possible mechanism of  suppression of plant-parasitic nematodes  by entomopathogenic nematodes."  retrieved from STN  XP002136524  abstract  &amp; NEMATOLOGY, (NOV., 1999) VOL. 1, NO.  7-8, PP. 735-743. ,</p>	1-9, 11, 29
X,P	<p>DATABASE BIOSIS 'Online!  BIOSCIENCES INFORMATION SERVICE,  PHILADELPHIA, PA, US  AN 2000:109391,  HAN, RICHOU ET AL: "Trans - specific  nematicidal activity of Photorhabdus  luminescens."  retrieved from STN  XP002136525  abstract  &amp; NEMATOLOGY, (NOV., 1999) VOL. 1, NO.  7-8, PP. 687-693. ,</p>	1-9, 11, 29
X,P	<p>CHEMICAL ABSTRACTS, vol. 132,  Columbus, Ohio, US;  abstract no. 163304,  HU, KAIJI ET AL: "Nematicidal metabolites  produced by Photorhabdus luminescens  (Enterobacteriaceae), bacterial symbiont  of entomopathogenic nematodes"  XP002136522  abstract  &amp; NEMATOLOGY (1999), 1(5), 457-469 ,</p>	1-9, 11, 29
X,P	<p>WO 99 42589 A (NOVARTIS ERFINDUNGEN  VERWALTUN ;NOVARTIS AG (CH); KRAMER VANCE  CAR) 26 August 1999 (1999-08-26)  cited in the application  page 1 -page 2, paragraph 3; claims</p>	12-18
X	<p>CHEMICAL ABSTRACTS, vol. 126,  Columbus, Ohio, US;  abstract no. 234686,  GEORGIS, R. ET AL: "Novel pesticidal  substances from the entomopathogenic  nematode-bacterium complex"  XP002136523  abstract  &amp; ACS SYMP. SER. (1997),  658(PHYTOCHEMICALS FOR PEST CONTROL),  134-143 ,</p>	1-9, 11, 29

-/--

## INTERNATIONAL SEARCH REPORT

International Application No

GB 00/00219

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. HU ET AL.: "Mortality of Plant-Parasitic Nematodes Caused by Bacterial ( <i>Xenorhabdus</i> spp. and <i>Photorhabdus Luminescens</i> ) Culture Media" JOURNAL OF NEMATOLOGY, vol. 27, no. 4, December 1995 (1995-12), XP000905673 the whole document	1-9, 11, 29
X	WO 98 08388 A (MORGAN JAMES ALUN WYNNE; JARRETT PAUL (GB); ELLIS DEBORAH JUNE (GB) 5 March 1998 (1998-03-05) cited in the application page 1, line 4 - line 9 page 6, line 21 -page 9, line 9 page 11, line 10 -page 12, line 19; claims 17,18,21,23-25,29	6-9, 11-30
X	PATENT ABSTRACTS OF JAPAN vol. 013, no. 286 (C-613), 29 June 1989 (1989-06-29) -& JP 01 080294 A (SUMITOMO CHEM CO LTD; OTHERS: 01), 27 March 1989 (1989-03-27) abstract; figures 2-1	12-18
A	WO 92 19739 A (MYCOGEN CORP) 12 November 1992 (1992-11-12) cited in the application	1-30
X	page 1 -page 2, line 32; claims	12-18

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

GB 00/00219

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9922598	A	14-05-1999	AU 9753098	A	24-05-1999
WO 9942589	A	26-08-1999	AU 3028699	A	06-09-1999
WO 9808388	A	05-03-1998	AU 4024997	A	19-03-1998
			CN 1233938	A	03-11-1999
			EP 0923295	A	23-06-1999
			ZA 9707373	A	15-02-1999
JP 01080294	A	27-03-1989	JP 2117839	C	06-12-1996
			JP 8017707	B	28-02-1996
WO 9219739	A	12-11-1992	AU 667041	B	07-03-1996
			AU 2025292	A	21-12-1992
			AU 656726	B	16-02-1995
			AU 7826491	A	12-12-1991
			BR 9205969	A	26-07-1994
			CA 2042868	A	12-12-1991
			EP 0462721	A	27-12-1991
			EP 0517367	A	09-12-1992
			HU 62928	A	28-06-1993
			JP 4229170	A	18-08-1992
			NZ 242560	A	26-01-1994
			PL 290626	A	24-02-1992
			US 5439881	A	08-08-1995
			US 5753492	A	19-05-1998
			ZA 9203168	A	27-01-1993

PCT

D INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



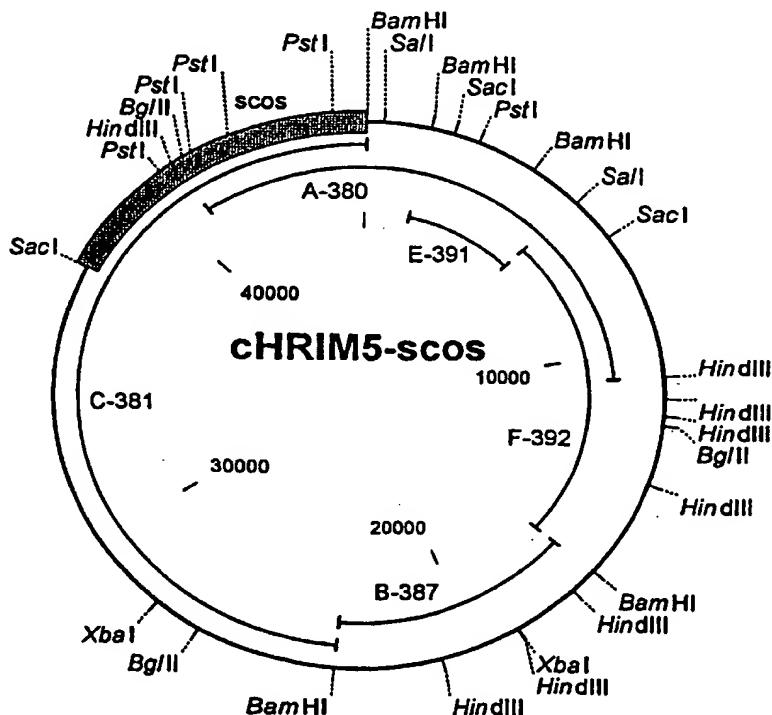
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : A01N 63/00, 63/02, C12N 15/31, C12P 21/00, C07K 14/24 // (C12P 21/00, C12R 1:01)		A1	(11) International Publication Number: <b>WO 00/42855</b>
			(43) International Publication Date: 27 July 2000 (27.07.00)
(21) International Application Number: PCT/GB00/00219		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 24 January 2000 (24.01.00)			
(30) Priority Data: 9901499.5 22 January 1999 (22.01.99) GB			
(71) Applicant (for all designated States except US): HORTICULTURE RESEARCH INTERNATIONAL [GB/GB]; Wellesbourne, Warwick, Warwickshire CV35 9EF (GB).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): MORGAN, James, Alun, Wynne [GB/GB]; Horticulture Research International, Wellesbourne, Warwick, Warwickshire CV35 9EF (GB). JARRETT, Paul [GB/GB]; Horticulture Research International, Wellesbourne, Warwick, Warwickshire CV35 9EF (GB). ELLIS, Debbie [GB/GB]; Horticulture Research International, Wellesbourne, Warwick, Warwickshire CV35 9EF (GB). OUSLEY, Margaret, Anne [GB/GB]; Horticulture Research International, Wellesbourne, Warwick, Warwickshire CV35 9EF (GB).		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.	
(74) Agent: RUFFLES, Graham, Keith; Marks & Clerk, 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).			

(54) Title: BIOLOGICAL CONTROL OF NEMATODES

(57) Abstract

Nematodes can be controlled through the use of bacteria associated symbiotically with an entomopathogenic nematode. The bacteria can be employed for nematode control, or engineered to a recombinant form. Control may be achieved using material such as a peptide. The peptide can be obtained from a natural or engineered nucleic acid.





**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## BIOLOGICAL CONTROL OF NEMATODES

## TECHNICAL FIELD

The present invention relates to methods and materials for controlling nematodes.

## PRIOR ART

Several thousand species of nematodes, sometimes called eel worms, are known. Numerous nematodes attack and parasitize humans and animals and cause disease. Additionally, several hundred species are known to feed on living plants. Certain of these are reviewed by Agrios in "Plant Pathology - 3rd Ed" Pub Academic Press Inc, see Chapter 15 therein.

Methods of controlling nematodes and their associated diseases include cultural practices; biological methods, e.g. use of resistant varieties; physical methods, e.g. heat; and use of chemical agents.

Patent application WO 92/19739 (Mycogen) relates to genes and gene fragments from *Bacillus thuringiensis* which have nematocidal activity. These generally encode crystal toxins from particular strains.

Patent application EP 0 303 426 (Mycogen) also relates to strains of *B. thuringiensis* which have nematocidal activity.

Patent application EP 0 171 381 (Monsanto) relates to particular soil bacteria which are capable of proliferating in an environment which is infested with

nematodes such as pseudomonads which colonise the surface of plant roots. The basis for the controlling activity appears to stem from glycosidase enzymes which are hypothesised to directly inhibit the nematodes.

Notwithstanding these disclosures, there is an ongoing requirement for materials which have nematocidal activity, for instance for use in crop protection or nematode-mediated disease control.

Patent application PCT/WO 99/22598 (University of Reading) published 14 May 1999 claims a biopesticide for the control of insect pests or plant parasitic nematodes or both, which comprises as an effective agent a species of bacteria which is a symbiont of an entomopathogenic nematode.

#### DISCLOSURE OF THE INVENTION

The present inventors have established that species of bacteria which in nature are associated symbiotically with entomopathogenic nematodes, can in fact be utilised to control nematodes, and in preferred forms of the invention, to kill them. The bacteria themselves can be employed, or nematode control agents can be used which are derived from such bacteria. In one aspect of the invention, the present invention employs bacteria which are engineered and thus not naturally occurring, or nematode control agents which are derived from natural or non-natural bacteria.

It has been reported that certain bacterial species such as *Xenorhabdus* and *Photorhabdus* can be used to control insects, see e.g. PCT/WO 98/08388 of MAFF, PCT/WO 97/17432 of WARF, and PCT/WO 99/42589 of Novartis. An effect against nematodes had not previously been demonstrated.

The symbiotic bacteria used in the present invention are isolatable from nematodes or the insects which the nematodes attack, and differ fundamentally in terms of life-style and activity from those soil bacteria such as *B. thuringiensis* or pseudomonads which have previously been suggested as being nematocidal.

Indeed, *prima facie*, it seems highly unlikely that nematode symbiotes might possess nematocidal activity. However, in the light of the present disclosure, a number of possible explanations for the observed activity can be tentatively proposed. Firstly, in order to protect a nutrient supply from a dead insect, the bacteria might produce anti-nematocides to prevent saprophytic nematodes gaining access. Alternatively, to become a symbiont, the bacterial strains may have once been pathogens of these nematodes and evolved towards a less hostile symbiotic relationship. The nematocidal activity may be an evolutionary throwback from the original pathogenic relationship, in which case it may be expected to be widely present amongst bacteria which have evolved in this way.

A first aspect of the present invention is the use of bacterial strains to control a target nematode, characterised in that in nature the bacterial strain is associated symbiotically with an entomopathogenic nematode.

As discussed in more detail below, the bacterial strains may be used in the methods of the present invention *per se*, or they may be used as a source of nematode control agent. The nematode control agent can be derived directly, or be prepared and utilised through recombinant DNA techniques, optionally via a host cell.

The target nematode will generally be different to the nematode with which the bacterial strain is found symbiotically in nature.

By means of the present invention employing bacteria or a nematode control agent, it becomes possible to control nematodes, in the sense of, to prevent or retard the effect that the nematode has on other organisms such as animals or more preferably plants, or to reduce the number of nematodes or nematode eggs in an area of interest, or to alleviate or cure a disease caused by nematodes. Control may be at the level of larval nematodes or nematode eggs, or may inhibit the motion, feeding or infectivity of adult nematodes. Nematocidal control may be employed to kill the nematode target. Such controlling activity can be assessed as shown in the Examples below.

#### PREFERRED EMBODIMENTS

The present invention provides a composition for the control of parasitic nematodes which comprises as an effective agent a species of bacteria which is a symbiont of an entomopathogenic nematode, or engineered bacteria having such activity, or a nematode control agent derived from natural or engineered bacteria.

Correspondingly, the present invention also provides a method of nematode control employing such a composition.

The bacterial species is typically of the genera *Xenorhabdus* or *Photorhabdus*, preferably the genus *Xenorhabdus*, for instance the species *Xenorhabdus bovienii*. Examples of particularly preferred bacteria include:

*Xenorhabdus bovienii* strain H31 deposited with NCIMB under accession number NCIMB 40985 on 20 January 1999;

*Xenorhabdus bovienii* strain I73 deposited with NCIMB under accession number NCIMB 40986 on 05 November 1998; and

*Xenorhabdus* strain C42 deposited with NCIMB under accession number NCIMB 41004 on 05 November 1998.

The nematode control agent can be a peptide derived from a symbiont of an entomopathogenic nematode or an engineered bacterium has functional activity against a nematode. The peptide nematode control agent can be produced from a nucleic acid derived from a symbiont of an entomopath nematode or an engineered bacterium and which encodes such a peptide. The peptide can be an oligopeptide or a polypeptide, notably a protein. In one version, the nematode control agent is a toxin with toxic activity against nematodes, but the nematode control agent can have other activity.

The nucleic acids of this invention can be employed in a method of producing a peptide comprising the step of causing or allowing the expression from a nucleic acid of this invention in a suitable host cell.

The nucleic acid can comprise a natural nucleotide sequence or a degeneratively equivalent sequence, and functional variants thereof. Variants include homologous variants encoding a peptide which is a nematode control agent, the nucleic acid having 70% or more DNA sequence identity and/or the peptide having 70% or more amino acid sequence identity. Especially preferred nucleic acids in p 13-1f and p 14-2f and variants thereof.

The present invention extends to nucleic acids having a sequence which is a derivative by way of addition, insertion, deletion or substitution of one or more nucleotides. The nucleic acid can contain longer expressed sequences such that the nematode control agent is expressed as a fusion protein.

Nucleic acids complementary to the nucleic acid encoding a nematode

control agent are also part of this invention.

Nucleic acids for use as a probe or primer having a nucleotide sequence of at least 15, 18, 21, 24 or 30 nucleotides, which sequence is present in, or complementary to, the nucleic acid encoding nematode control agent are further provided by this invention. In this respect, the invention extends to a method for identifying or cloning a nucleic acid for nematode control agent which method employs such a nucleic acid probe.

A method provided by this invention comprises the steps of:

- (a) providing a preparation of nucleic acid from a bacterium,
- (b) providing a probe,
- (c) contacting nucleic acid in said preparation with said probe under conditions for hybridisation of probe to any said gene or homologue in said preparation, and,
- (d) identifying said gene or homologue if present by its hybridisation with said probe.

The hybridisation conditions can be selected to allow the identification of sequences having 70% or more sequence identity with the probe.

In one embodiment, the method comprises use of two primers to amplify a nucleic acid encoding a nematode control agent, at least one of the primers having a conserved nucleotide sequence of at least 15 nucleotides.

A method is further made possible by this invention comprising the steps of:

- (a) providing a preparation of nucleic acid from a bacterium,
- (b) providing a pair of nucleic acid molecule primers, at least one of which is a primer,
- (c) contacting nucleic acid in said preparation with said primers under

conditions for performance of PCR,

(d) performing PCR and determining the presence or absence of an amplified PCR product.

Additionally, the invention provides a recombinant vector comprising a nucleic acid of this invention. The vector is preferably capable of replicating in a suitable host such as *E. coli* or in *Xenorhabdus*. The vector can be a baculovirus. In a preferred feature, the nucleic acid is operably linked to a promoter or other regulatory element for transcription in a host cell. Vectors can further comprise any one or more of the following: a terminator sequence; a polyadenylation sequence; an enhancer sequence; a marker gene; a sequence encoding pesticidal material derived from *Bacillus thuringiensis*.

The vector can be a plant vector.

The vector of this invention can be introduced into a cell. Thus, a method for transforming a plant cell comprises the step of causing or allowing recombination between the vector and the plant cell genome to introduce the nucleic acid into the genome. The nucleic acid can be incorporated into chloroplast DNA, or into mitochondrial DNA.

Host cells comprising a vector are also part of this invention. The host cell can be a plant cell, which may be in a plant.

To this end, a method for producing a transgenic plant comprises the step of regenerating a plant from the transformed cell. In turn, plants of this invention extend to the progeny of such plants.

Examples of plants of this invention include crop species which can be



protected, notably maize, cotton, soya, rice, *Brassica* species, tomato, potato, sugar beet, barley, soybean, peanut, onion, rye, wheat, corn, banana, raspberry, bean. Decorative and other plants are also possible, e.g. rose.

A part of the propagule of the plants is also envisaged by this invention.

A method of influencing or affecting the toxicity of a cell such as a plant cell is provided where the method includes causing or allowing expression of a heterologous nucleic acid of this invention within the cells.

In a further aspect, the invention involves the use of a material selected from: an *X. bovienii* strain, a nematode control agent; a nucleic acid; a host cell; a plant; a peptide; or a composition of the invention, for the control of a pest, especially where the pest is a nematode and the material is used to control the nematode.

The present invention extends to control of helminthiasis in humans and other animals including domesticated animals such as swine, sheep, horses, cattle, goats, dogs, cats and poultry. The nematodes to be controlled include *Haemonchus*, *Trichostrongylus*, *Ostertagia*, *Nematodirus*, *Cooperia*, *Ascaris*, *Bunostomum*, *Oesophagosromuni*, *Chabertia*, *Trichuris*, *Strongylus*, *Trichonema*, *Dictyocaulus*, *Capillaria*, *Heterkis*, *Toxocara*, *Ascaridia*, *Oxyuris*, *Ancylostoma*, *Uncinaria*, *Toxascaris*, *Caenorhabditis* and *Parascaris*.

Target nematodes may be selected from the genera *Aphelenochoides*, *Anguina*, *Bursaphelenchus*, *Criconemella*, *Melodigyne*, *Ditylenchus*, *Globodera*, *Heliocotylenchus*, *Heterodera*, *Pratylenchus*, *Radopholus*, *Rotelynychus*, *Tylenchus*, *Trichodorus*, *Xiphenema*, and *Caenorhabditis*.

The compositions of this invention can be used in conjunction with *Bacillus*

*thuringiensis* or pesticidal materials derived therefrom.

In a further aspect, there is provided an antibody or fragment thereof, or a polypeptide comprising the antigen-binding domain of the antibody, capable of specifically binding a peptide of this invention.

Such an antibody or fragment can be obtained by immunising a mammal with the peptide, and is useful in a method of identifying and/or isolating a nematode control agent comprising the step of screening candidate polypeptides with a polypeptide comprising the antigen-binding domain of the antibody of claim.

Some further aspects of preferred embodiments of the invention will now be discussed.

#### Bacterial strains

These can be derived from any entomopathogenic nematode. Preferred species are *Xenorhabdus* and *Photorhabdus*.

Potential sources of bacteria for use in the methods of the present invention may be identified by any preferred method. For instance, entomopathogenic nematodes can be isolated using an insect baiting technique such as that described by Bedding & Akhurst (1975) *Nematologia* 21: 215-227. Bacteria from nematodes identified as being pathogenic to the insect are isolated, cultured, and used as a source of nematocidal agent, e.g. by analogy with the methods used in the Examples below. Preferably *Xenorhabdus* or *Photorhabdus* species are used.

The preferred bacterial strains include ones which have the characteristics of

strain C42, I73 or H31 isolated by the present inventors. This *Xenorhabdus* strain has the following characteristics: rod shaped; motile; non-bio luminescent; blue on NBTA; produces antibiotics; resistant to ampicillin; forms circular colonies; has convex morphology; white colour.

This strain was presumptively identified as belonging to the genera *Xenorhabdus* since it was isolated from an insect killed by an entomopathogenic nematode and had the above characteristics. The strain has been deposited at the NCIMB (23 St Machar Drive, Aberdeen, AB24 3RY, Scotland) by the applicants under accession number NCIMB 41004 on 20 January 1999.

Further preferred strains of the present invention are two strains of *X. bovienii* designated H31 and I73 which have also been deposited under the terms of the Budapest Treaty at the NCIMB under the accession numbers NCIMB 40985 and 40986 respectively. These share characteristics of C42 in that they are rod-shaped; motile; non-bioluminescent; blue on NBTA; produce antibiotics; resistant to ampicillin; form circular colonies; and have convex morphology. The strains were identified as belonging to the species *X. bovienii* when compared to the *X. bovienii* type strain T228 using Restriction Analysis of the complete 16S rRNA gene and partial sequence analysis.

#### Target nematodes and diseases

The group of diseases described generally as helminthiasis is due to infection of an human or other animal host with parasitic worms known as helminths. Helminthiasis is a prevalent and serious economic problem in domesticated animals such as swine, sheep, horses, cattle, goats, dogs, cats and poultry. Among the helminths, the group of worms described as nematodes causes

widespread and often at times serious infection in various species of animals. The most common genera of nematodes infecting the animals referred to above are *Haemonchus*, *Trichostrongylus*, *Ostertagia*, *Nematodirus*, *Cooperia*, *Ascaris*, *Bunostomum*, *Oesophagostomum*, *Chabertia*, *Trichuris*, *Strongylus*, *Trichonema*, *Dictyocaulus*, *Capillaria*, *Heterakis*, *Toxocara*, *Ascaridia*, *Oxyuris*, *Ancylostoma*, *Uncinaria*, *Toxascaris*, *Caenorhabditis* and *Parascaris*. Certain of these, such as *Nematodirus*, *Cooperia*, and *Oesophagostomum*, attack primarily the intestinal tract, while others, such as *Dictyocaulus* are found in the lungs. Still other parasites may be located in other tissues and organs of the body.

The bacteria and encoded toxins of the invention may be used as nematocides for the control of the nematodes and diseases discussed above. More preferably, however, they are used to control soil and plant parasitic nematodes. Particular crop species which can be protected include tomatoes, potatoes, sugar beet, barley, soybean, peanut, onion, rye, wheat, corn, banana, raspberry, beans. Decorative and other plants may also be treated e.g. rose.

Target nematodes may be selected from the genera *Aphelenchoides*, *Anguina*, *Bursaphelenchus*, *Crictonemella*, *Meloidogyne*, *Ditylenchus*, *Globodera*, *Helicotylenchus*, *Heterodera*, *Pratylenchus*, *Radopholus*, *Rotelynchus*, *Tylenchus*, *Trichodorus*, *Xiphenema*. A further organism used in certain of the Examples below is *Caenorhabditis elegans*. Other target organisms and plants are discussed by Agrios in "Plant Pathology - 3rd Ed" Pub Academic Press Inc, see Chapter 15 therein.

As stated above, the target nematode will generally be different to that with which the bacterial strain is found in nature.

## Methods of use of bacteria

The bacteria may be used in any appropriate method which brings them into contact with the target nematode, preferably such that they, or their products, are ingested or absorbed by the target nematode.

In particular, regarding plants, the bacteria may be formulated in a variety of ways so as to enhance stability. For instance they may be employed in admixture with substrates to protect the cells.

The mixture can be spread over, ploughed into or otherwise mixed with nematode infected or potentially infected soil.

Regarding animals, bacteria intended for enteric inoculation can be mixed with carrier material that is suitable for ingestion by the intended animals.

## Isolation of agent

Nematode control agents of the present invention, which may be proteinaceous, or nucleic acids encoding them, may be isolated and/or purified from the C42, I73 or H31 bacteria described above, in substantially pure or homogeneous form, or free or substantially free of other materials from the bacterial strain of origin. Where used herein, the term "isolated" encompasses all of these possibilities.

Methods of purifying proteins from heterogenous mixtures are well known in the art, e.g. selective precipitation, proteolysis, ultrafiltration with known molecular weight cut-off filters, ion-exchange chromatography, gel filtration, etc. A particularly useful initial technique in this regard is ultracentrifugation. Further methods which are known to be suitable for

protein purification are disclosed in "Methods in Enzymology Vol 182 - Guide to Protein Purification" Ed. M P Deutscher, Pub. Academic Press Inc. Other references which outline techniques commonly used by those of ordinary skill in the art include "Protein Purification - principles and practice" Pub. Springer-Verlag, New York Inc (1982), and by Harris & Angal (1989) "Protein purification methods - a practical approach " Pub. O.U.P. UK.

Nematocidal activity may be assessed using a spread assay as discussed below.

The C42, I73 or H31 agent may be wholly or partially synthetic. In particular they may be recombinantly produced from nucleic acid sequences which are not found together in nature (do not run contiguously) but which have been ligated or otherwise combined artificially.

For instance, in the Examples below, nucleic acid encoding toxin(s) from I73 has been expressed in hosts cells using a vector system. Amino acid sequences of 38 different putative I73 toxin(s) are set out in sequence Annex I. These sequences are based on the nucleic acid sequence set out in Fig 2 ('chrim5'), a cosmid clone derived from I73 genomic DNA which conferred nematocidal activity upon *E. coli* cells into which it was introduced (i.e. significantly reduced nematode larval growth and development, and feeding). As detailed below, the entire amino acid sequence as set out in each case may not be required for nematocidal activity. In particular the portion up to the first Met in each sequence may be omitted, as may other portions which may not contribute to the nematocidal activity. Thus, not all the proteins or genes may be required for nematocidal activity, and usually there will be one or more principal proteins, though others may play supporting roles such as in enhancing the activity or encoding other nematocidal activities.

Thus isolated nematocidal agents comprising a polypeptide containing all, or a nematocidal fragment, of any of the depicted I73 sequences, form one aspect of the present invention. Preferred agents include those encoded by p14-2f and p13-1f. Other active variants of these sequences are also encompassed as described below.

Candidate agents for use in this invention to control nematodes extend to those from the bacteria described in PCT/WO 99/22598, as well as the insecticidal toxins and bacteria of PCT/WO 99/42589, PCT/WO 98/08388 and PCT/WO 97/17432, the disclosures of which are incorporated by reference.

#### Nucleic acids and variants

In one aspect of the present invention there is provided a nucleic acid molecule encoding a nematode control agent of the present invention, for example a toxin, as described above.

The nucleic acid may be derived from the sequence shown in Fig 2 or the complement (or degenerate equivalent) thereof. This sequence (cHRIM5) was itself derived from I73 and identified by its unexpected nematocidal activity. Regions of this sequence believed to correspond to genes of the present invention are described in Fig 3. Isolated nucleic acids comprising one or more of these regions which encode a nematocidal activity are particularly preferred.

In the light of the present disclosure, further nucleic acids of the present invention may be isolated using PCR or southern blotting or other techniques well known to those skilled in the art. This requires the use of two primers to specifically amplify target nucleic acid, so preferably two

nucleic acid molecules with sequences characteristic of the C42, H31 or most preferably an I73 toxin isolated as above are employed. Using RACE PCR, only one such primer may be needed (see "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990)).

Thus a method involving use of PCR in obtaining nucleic acid according to the present invention may include:

- (a) providing a preparation of bacterial nucleic acid,
- (b) providing a pair of nucleic acid molecule primers suitable for PCR, at least one of said primers being a primer based on a toxin from C42, H31 or I73,
- (c) contacting nucleic acid in said preparation with said primers under conditions for performance of PCR,
- (d) performing PCR and determining the presence or absence of an amplified PCR product. The presence of an amplified PCR product may indicate identification of a variant.

In a further aspect of the present invention there are disclosed nucleic acids which are variants of the C42, I73 or H31 toxin. A variant nucleic acid molecule shares homology (or identity) with all or part of the C42, H31, or most preferably I73 sequence discussed above.

Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. Methods in Enzymology 183: 63-98). Parameters are set, using the default matrix blosum62, as follows:

Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA

Gapext (penalty for additional residues in a gap): -2 for proteins / -4 for DNA

KTUP word length: 2 for proteins / 6 for DNA.



Homology (similarity or identity) may be at the nucleotide sequence and/or encoded amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares at least about 70%, 75%, 80%, or 85% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology.

Another method for assessing homology at the nucleic acid level is by hybridization screening. One common formula for calculating the stringency conditions required to achieve hybridisation between nucleic acid molecules of a specified sequence homology is shown in Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press:

$T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41 (\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\text{\#bp}$   
in duplex

As an illustration of the above formula, using  $[\text{Na}^+] = [0.368]$  and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the  $T_m$  is  $57^{\circ}\text{C}$ . The  $T_m$  of a DNA duplex decreases by 1 -  $1.5^{\circ}\text{C}$  with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of  $42^{\circ}\text{C}$ . Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

Variants of the present invention can be artificial nucleic acids.

Alternatively they may be novel, naturally occurring, nucleic acids, isolatable using the information disclosed herein. Thus a variant may be a distinctive part or fragment (however produced) corresponding to a portion of the C42, I73 or H31 toxin. The fragments may encode particular functional parts of the agent or they may be used for probing for, or amplifying, sequences corresponding to C42, I73 or H31 toxin. Sequence variants which occur naturally may include homologs of the C42, I73 or H31 toxin from other

bacteria, including nematode-symbionts. Artificial variants (derivatives) may be prepared by those skilled in the art, for instance by site directed or random mutagenesis (i.e. nucleotide addition, deletion or substitution, optionally to lead to amino acid addition, deletion or substitution) or by direct synthesis. Preferably the variant nucleic acid is generated either directly or indirectly from an original nucleic acid encoding the C42, I73 or H31 toxin.

Changes may be desirable for a number of reasons, including introducing or removing the following features. Sites which are required for pre- or post-translation modification. Changes for codon usage preferences to enhance gene expression in different organisms. Leader or other targeting sequences (e.g. membrane or golgi locating sequences) may be added to the expressed protein to determine its location following expression. All of these may assist in efficiently cloning and expressing an active polypeptide in recombinant form. Other desirable mutation may be random or site directed mutagenesis in order to alter the activity (e.g. host specificity) or stability of the encoded polypeptide. Changes may be by way of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Also included are active (nematocidal) variants having non-conservative substitutions.

Variant nucleic acids encompass all of these possibilities. When used in the context of polypeptides or proteins they indicate the encoded expression product of the variant nucleic acid i.e. variants of C42, I73 or H31 toxin e.g. variants of the I73 toxin sequences disclosed hereinafter.

Vectors and production of host cells

In one aspect of the present invention, the nucleic acid encoding the nematode control agent is provided in the form of a recombinant and preferably replicable vector.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Sambrook et al (1989) *supra*.

The permitted vectors include, *inter alia*, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally, e.g. an autonomous replicating plasmid with an origin of replication. Illustratively integration can occur into chloroplast DNA or into mitochondrial DNA.

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate optionally inducible promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, yeast, filamentous fungal or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell. The vectors and host cells into which they are introduced may be used to clone or otherwise

identify nucleic acids according to the invention.

The agent may be used as part of a viral vector which is itself pathogenic to nematodes.

Also of interest in the present context are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148). Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809). Suitable promoters which operate in plants include the Cauliflower Mosaic Virus 35S (CaMV 35S). Other examples are disclosed at page 120 of Lindsey & Jones (1989) "Plant Biotechnology in Agriculture" Pub. OU Press, Milton Keynes, UK.

#### Host cells

The toxin genes or gene fragments encoding the nematocidal agents of the subject invention may be introduced into a host cell, microbial, animal or plant. Expression of the toxin gene in the host cell results, directly or indirectly, in the intracellular production and maintenance of the nematocide.

Thus the present invention also provides methods comprising introduction of such a construct into a plant cell or a microbial cell and/or induction of expression of a construct within a cell, by application of a suitable stimulus e.g. an effective exogenous inducer.

Hosts may be used to assay the activity of particular sequences or

fragments. Hosts can also be used to generate quantities of toxin which can be employed in situ in suitable treated cells, or alternatively with suitable hosts, e.g., *Pseudomonas* viable microbes can be applied to the sites of nematodes where they will proliferate and where they or their products can be ingested by the nematodes. Higher organisms, preferably plants, can also be engineered with the toxin. The result in each case is a control of the nematodes. A host may be selected that can tolerate harsh environmental conditions and then grow when they improve, as illustrated by *Bacillus* species where the spores can exist under environmental extremes.

Characteristics of interest for use as a nematocide microcapsule i.e. a vehicle for the active agent include protective qualities for the nematocide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to nematodes for ingestion; ease of killing and fixing without damage to the toxin; and the like.

#### Treated host cells

Where the cell is treated, the cell will usually be intact and be substantially proliferative form when treated, rather than in a spore form, although in some instances spores may be employed. Treatment of the microbial cell, e.g. a microbe containing the bacterial toxin gene or gene fragment, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability in protecting the toxin.

#### Viable hosts

Where the toxin gene or gene fragment is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is preferable that microorganism hosts are selected which are known to occupy the phytosphere (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the nematocide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilus*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconosroc*, and *Alcaligenes*; fungi, particularly yeast, e.g., genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodororula*, and *Aureobasidium*.

#### Plants as hosts

Nucleic acid encoding the nematocides of the present invention can be introduced into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882,

EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) Plant Tissue and Cell Culture, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. Plant Cell Physiol. 29: 1353 (1984)), or the vortexing method (e.g. Kindle, PNAS U.S.A. 87: 1228 (1990d). Physical methods for the transformation of plant cells are reviewed in Oard, 1991, Biotech. Adv. 9: 1-11.

*Agrobacterium* transformation is widely used by those skilled in the art to transform dicotyledonous species. It has also been used with filamentous fungi (see de Groot et al, 1998, Nature Biotechnology 16: 839-842).

Recently, there has also been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (see e.g. Hiei et al. (1994) The Plant Journal 6, 271-282)). Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* alone is inefficient or ineffective.

Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g. bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

Generally speaking, following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications, Academic Press, 1984, and Weissbach and

Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989.

The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) Current Opinion in Biotechnology 5, 158-162.; Vasil, et al. (1992) Bio/Technology 10, 667-674; Vain et al., 1995, Biotechnology Advances 13 (4): 653-671; Vasil, 1996, Nature Biotechnology 14 page 702).

#### Combination nematocides

In further embodiments of the invention, bacteria associated with entomopathogenic nematodes or the toxins or products discussed above are used in conjunction with other nematocidal bacteria such as *B. thuringiensis* strains (e.g. from WO 92/19739) or pesticidal materials derived therefrom.

#### Materials for use in the present invention

The present invention also embraces materials for use in the methods above. These materials include the novel bacterial strains which are associated symbiotically with an entomopathogenic nematode and which are capable of controlling a target nematode. In particular the invention encompasses strain C42, I73 or H31 in isolated or substantially isolated form, or strains having the characteristics of C42, I73 or H31 (including nematocidal activity assessed as below).

Also embraced are compositions and formulations of these bacteria. These may comprise or consist of wettable powders, granules or dusts, mixed with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, methylcellulose, xanthan gum and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells,



peat moss, vermiculite, soil, seeds, other plant tissue and the like). The formulations may include spreader-sticker adjutants, stabilizing agents or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

Bacteria may be mixed with other material while in freeze-dried form, encapsulated in biodegradable or water-soluble material, or otherwise treated to prolong their viability or decrease their levels of metabolic activity during handling. If desired, the carrier material may contain assimilable nutrient sources to support proliferation of the bacteria.

Also included are purified or substantially purified nematocidal agents (particularly proteinaceous agents) isolated or isolatable from the strains or host cells discussed above.

Thus the invention further discloses nematocidal compositions comprising one or more agents as described above. Such compositions preferably further comprise other nematocidal materials from other *Xenorhabdus* species or non-*Xenorhabdus* species. These other materials may be chosen such as to have complementary properties to the agents described above, or act synergistically with it.

Toxins of the invention for use with animals can be adapted to be administered orally in a unit dosage form such as a capsule, bolus or tablet, or as a liquid drench when used as an anthelmintic in mammals, and in the soil to control plant nematodes. The drench is normally a solution, suspension or dispersion of the active ingredient, usually in water, together with a suspending agent such as bentonite and a wetting agent or like

excipient. Generally, the drenches also contain an antifoaming agent. Drench formulations generally contain from about 0.001 to 0.5% by weight of the active compound. Preferred drench formulations may contain from 0.01 to 0.1% by weight, the capsules and boluses comprise the active ingredient admixed with a carrier vehicle such as starch, talc, magnesium stearate, or dicalcium phosphate. Where it is desired to administer the toxin compounds in a dry, solid unit dosage form, capsules, boluses or tablets containing the desired amount of active compound usually are employed. These dosage forms are prepared by intimately and uniformly mixing the active ingredient with suitable finely divided diluents, fillers, disintegrating agents and/or binders such as starch, lactose, talc, magnesium stearate, vegetable gums and the like. Such unit dosage formulations may be varied widely with respect to their total weight and content of the antiparasitic agent, depending upon the factors such as the type of host animal to be treated, the severity and type of infection and the weight of the host.

When the active compound is to be administered via an animal feedstuff, it is intimately dispersed in the feed or used as a top dressing or in the form of pellets which may then be added to the finished feed or, optionally, fed separately. Preferably, a carrier for feed administration is one that is, or may be, an ingredient of the animal ration. Suitable compositions include feed premixes or supplements in which the active ingredient is present in relatively large amounts and which are suitable for direct feeding to the animal or for addition to the feed either directly or after an intermediate dilution or blending step. Typical carriers or diluents suitable for such compositions include, for example, distillers' dried grains, corn meal, citrus meal, fermentation residues, ground oyster shells, wheat shorts, molasses solubles, corn cob meal, edible bean mill feed, soya grits, crushed limestone and the like.

Alternatively, the antiparasitic compounds may be administered to animals parenterally, for example, by intraluminal, intramuscular, intratracheal, or subcutaneous injection, in which event the active ingredient is dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material is suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety, such as peanut oil, cotton seed oil and the like. Other parenteral vehicles, such as organic preparations using solketal, glycerol, formal and aqueous parenteral formulations, are also used. The active compound or compounds are dissolved or suspended in the parenteral formulation for administration; such formulations generally contain from 0.005 to 5% by weight of the active compound.

Further aspects of the invention include nucleic acids, vectors and host cells containing a heterologous construct according to the present invention, especially a plant or a microbial cell.

Such microbial cells may be treated as described in the methods above. Examples of chemical reagents are halogenating agents. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Bouin's fixative and Helly's fixative (See: Humason, Gretchen L., Animal Tissue Techniques, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host animal. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the nematode control agent.

In all of the compositions discussed above, the nematocide concentration may vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The nematocide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the nematocide while the liquid formulations will generally be from about 16% by weight of the solids in the liquid phase. The formulations will generally have from about  $10^2$  to about  $10^{10}$  cells/mg, more preferably  $10^7$  to about  $10^9$  cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare. The formulations can be applied to the environment of the nematodes, e.g., plants, soil or water, by spraying, dusting, sprinkling, or the like.

In addition to the above the invention includes plant cells which have been transformed with the genes of the present invention, and plants which include such plant cells.

#### EXAMPLES OF THE INVENTION

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

#### FIGURES

Fig 1 shows the cHRIM5 cosmid vector and subclones used for sequencing, as described in Example 6.

Fig 2 shows the sequence of cHRIM5 (1-37544 bps).

Fig 3 shows the position and orientation of ORFs in the cHRIM5 sequence.

Fig 4 shows deletions of cHRIM5 tested for nematocidal activity.

Fig 5 illustrates cloning of nematocidal activity in PLEX.

#### Example 1 - Source of strains C42, I73 and H31

Strain C42 was obtained using an insect entrapment method. Insects which were killed on the surface of a soil sample were observed under a microscope at high magnification. Any that contained high numbers of bacteria and not fungal hyphae were presumed to have been killed by insect parasitic nematodes. The identified presence of nematodes also aids this identification step, but it is not essential. These samples were plated on to NBTA media (see Poinar & Thomas, 1984 Nematodes p238-280 in "Laboratory guide to insect pathogens and parasites" Eds. Poiner & Thomas, Pub. Plenum Press, New York). Any colonies that developed that had characteristic features (e.g. morphology, size, colour) of *Xenorhabdus* or *Photorhabdus* strains were selected. Non-luminescent colonies were presumptively identified as *Xenorhabdus*. The identity of those having nematocidal activity as assessed in Example 3, is further confirmed using 16s rRNA sequence data (see Brunel et al 1997, Applied and Environmental Microbiology 63,2: 574-580).

I73 and H31 strains were obtained in a similar way to strain C42 but they were identified as belonging to the species *X. bovienii* when compared to the *X. bovienii* type strain T228 using Restriction Analysis of the complete 16S

rRNA gene (see Brunel et al, 1997 Applied and Environmental Microbiology: 574-580), and partial 16s ribosomal RNA sequence analysis.

#### Example 2 - Cell growth and preservation

Subcultures of the *Xenorhabdus* species C42, I73 and H31 were used to inoculate three 9 cm diameter petri dishes containing L agar (10g tryptone, 5 g Yeast Extract, 5 g NaCl and 15 g agar per lt). Plates were incubated for 48 hrs at 26°C and the resulting growth harvested by scraping off bacterial cells and thoroughly resuspending in 40 mls of 5% w/v lactose. The cells were washed once by centrifugation (5000 x *g* for 10 mins), resuspended in 10 mls of 5% w/v lactose, dispensed into 1 ml aliquots and freeze dried (-60°C for 48 hrs ) for medium term storage at 2°C. Other stocks were re-suspended in nutrient broth containing 10% w/v glycerol (Protect) and frozen at -70°C.

#### Example 3- Activity of cells against *Caenorhabditis elegans*

The bioassays were performed by allowing *C. elegans* to feed on live bacterial cell suspensions spread over the surface of Luria broth agar (Luria broth containing 1.2%w/v agar) in segmented square petri dishes (2.0 x 2.0 cm per test well). A minimum of three test wells, each containing 50-100 nematodes were used for each test. Mortalities were recorded after 3 days at 18°C.

*C. elegans* was cultured on *Escherichia coli* at 18°C on 9 cm diameter LB agar plates. Once the nematodes had colonised the complete plate they were re-subbed on a fresh plate to maintain stocks and the remainder re-suspended in 40 ml LB. The tube was allowed to stand for 15 min and the nematodes settled to the bottom. The concentrated nematodes were removed using a

sterile pipette and placed in 40 mls of fresh LB. The process was repeated 5 more times to wash the nematodes away from the *E. coli* cells. The nematodes were then diluted so that approximately 50 nematodes were present in 50 µl of LB.

The *Xenorhabdus* cells used were cultured in LB at 30°C/100 rpm for 24 hours and 50 µl spread on to the surface of each test well. The control *E. coli* cells were treated in a similar way but incubated at 37°C for growth. After application the wells were air dried for 30 min, and 50 µl of the nematode suspension placed in each well. Again the wells were air dried for 30 min. Plates were incubated at 18°C with 80% relative humidity for 3 days.

*Xenorhabdus* spp. C42, H31 and I73 gave 95% mortality, as compared with no significant effect for certain other *Xenorhabdus* bacterial strains and *E. coli*. Thus these results clearly show that cells from *Xenorhabdus* C42, H31 and I73 are an effective nematocide.

#### Example 4 - Cloning of nematode active gene from I73

Total DNA was isolated from I73 using a Quiagen genomic DNA purification kit (cat no. 10243). To isolate DNA, cells were grown in Luria broth (10g tryptone, 5g yeast and 5g NaCl per lt) at 26°C with shaking at 200 rpm to an optical density of 1.5 A600. Cells were harvested by centrifugation at 4000 x g and the DNA isolated using Quiagen 100/G tips, as per manufacturer's instructions. The purified DNA was stored at -20°C in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

To obtain a representative I73 library, total DNA was partially digested with *Sau3A*. Approximately 25 µg of DNA was incubated at 37°C with 0.25 units

of enzyme. At intervals of 5, 15, 30, 45 and 60 minutes, samples were removed and heated at 65°C for 10 minutes. To determine the size of the resulting DNA fragments, the samples were separated on a 0.5% (w/v) agarose gel. The samples containing a dominant DNA fragment size of between 30 and 50 Kb were combined and treated with shrimp alkaline phosphatase (Boehringer) for 20 minutes at 37°C. The DNA was ligated into the *Bam*HI site of the Stratagene cosmid vector Supercos1 (scos) and packaged into the *Escherichia coli* strain XL Blue 1, using a Gigapack II packaging kit (Stratagene) following the manufacturer's instructions.

To identify individual cosmid clones with activity to *C. elegans*, single colonies were grown in individual wells of segmented square petri dishes on Luria agar, containing 50 µg/ml ampicillin at 30°C for 24 hours. To each well, approximately 50, mainly L4 and adult *C. elegans* larvae were added in 50 µl of Luria broth. The dishes were incubated at 18°C and examined after 6 days for nematode development.

A total of 600 clones were examined and one coded cHRIM5 was found, which caused significant reduction in larval numbers, with no live L4 and adult larvae observed compared to on average, greater than 40 in all other clones tested.

#### Example 5 - Activity of cHRIM5, C42, H31 and I73 against *C. elegans*

Clone cHRIM5 was grown in 50 mls LB containing 50 µg of ampicillin per ml at 30°C/200 rpm for 40 hours. C42, H31 and I73 were grown in 50 mls LB at 26°C for 48 hours/200 rpm. Cultures were centrifuged at 4000 x *g* for 10 minutes, washed once and resuspended in 5 mls of PBS (0.05 mM phosphate buffer, 0.125M NaCl). To determine activity, 300 µl of cells were added in triplicate, to 1.2 ml of PBS containing 25, mainly L4 and adult *C.*



*elegans* larvae in multi well square dishes. As a control, an equivalent amount of XL 1 Blue *E. coli* cells containing Supercos 1 were used to determine nematode survival. The assays were incubated at 18 °C for 7 days before approximate nematode counts and observations were made.

#### Activity of cells on *C. elegans*

Cell line	No. and size of larvae/square	Cell turbidity
XL 1 Blue/Supercos 1	>100 (all stages)	Clear
XL 1 Blue/cHRIM5	<20(mainly small, L1,2 &3)	Cloudy
C42	<10	Cloudy
H31	<10	Cloudy
I73	<10	Cloudy

Thus cHRIM5, C42, H31 and I73 all gave a reduction in nematode numbers, and in particular cHRIM5 cells significantly reduced larval growth and development. All four strains caused a reduction in feeding (as indicated by the cloudy cell suspensions).

#### Example 6 - DNA and protein sequences

Plasmid and cosmid DNA for cloning was prepared using the QIAGEN midi system (tip 100, cat. No 12143). Cells were grown in Luria broth (Merck) at 37°C with shaking at 200 rpm for 18 hours. Cells were harvested by centrifugation at 6,000 x *g* and the DNA isolated as per manufacturers instructions. Restriction digestion (Roche, Life Technologies), dephosphorylation (Roche) and ligation (Life Technologies) were carried out using manufacturer's recommended conditions and as outlined by Sambrook et al. Transformation was accomplished using electrocompetant cells and a

BIO-RAD Gene pulser set at 12.5V cm<sup>-2</sup>. Two µl of DNA was used to electroporate 80 µl of early log phase *E. coli* DH5 alpha cells washed 3 times in sterile water (centrifugation at 6000 x g for 5 mins) and resuspended in 1/100th the original volume in 10% (v/v) glycerol. Luria agar containing either kanamycin or ampicillin at 50 µg ml<sup>-1</sup> were used to select clones where appropriate.

DNA sequence analysis of cHRIM5 was completed by sequencing a number of sub clones and primer walking, see figure 1 for the supercos vector, where the numbers are kbp. The sub clones used are as follows:

code	cHRIM5 treatment	vector used or remaining
A-380	<i>Hind</i> III digestion and self-ligation	deleted scos
B-387	<i>Bam</i> HI digestion and self-ligation	pUC 19- <i>Bam</i> H1 digestion
C-381	<i>Sal</i> I- <i>Bam</i> HI digestion	scos
E-391	<i>Sal</i> I- <i>Bam</i> HI digestion	pUC 19- <i>Sal</i> I <i>Bam</i> H1 digestion
F-392	<i>Sal</i> I- <i>Bam</i> HI digestion	pUC 19- <i>Sal</i> I <i>Bam</i> H1 digestion

Sub clone A-380 was constructed by digesting cHRIM5 DNA with the restriction enzyme *Hind*III and re-ligating fragments, this clone contains a deletion of the insert and scos cosmid DNA as the vector. Sub clone B-387 is a *Bam*HI digestion of cHRIM5 cloned into the plasmid pUC19 also cut with *Bam*HI and dephosphorylated. Sub clone C-381 was obtained by digesting cHRIM5 DNA with *Bam*HI and re-ligating the fragments, this clone contains the scos cosmid as the vector. Clones E-391 and F-392 were obtained by cutting cHRIM5 DNA with *Sal*I and *Bam*HI and ligating these fragments into the vector pUC19 also cut with these enzymes.

Sequencing was conducted using the artificial transposon AT2 (supplied by Perkin-Elmer-Applied Biosystems, Primer Island Transposition kit, cat No.

403015) using the cosmid cHRIM5 and all sub-clones as target DNA. One  $\mu\text{g}$  of cHRIM5 DNA was incubated with the transposon AT2 for 1 hour at  $30^\circ\text{C}$  in a final volume of  $20\ \mu\text{l}$ . After incubation the reaction was stopped by adding  $5\ \mu\text{l}$  of  $0.25\text{M}$  EDTA,  $1\%$  (w/v) SDS, and heat treatment at  $65^\circ\text{C}$  for 30 mins. The DNA was desalted by dialysis against water. One  $\mu\text{l}$  of the reaction mix was used to electroporate  $80\ \mu\text{l}$  of early log phase *E. coli* DH5 alpha cells. Colonies were selected on LB media containing  $50\ \mu\text{g/ml}$  trimethoprim. Once inserted the transposon mutants were used to provide a range of positions of primer sites at random intervals throughout the clones. The two primers PI+ and PI- near the end of the transposon were used to generate sequence data. In addition standard primers for the pUC19 and scos vectors were used to generate sequence data at the ends of each clone. DNA for sequencing was prepared using the QIAGEN ion exchange media (qiawell8, cat. No. 17122). Clones were grown in  $1\ \text{ml}$  of Luria broth containing trimethoprim ( $50\ \mu\text{g ml}^{-1}$ ) for 18 hours. Cells were centrifuged at  $13,000 \times g$  for 5 mins and resuspended in  $350\ \mu\text{l}$  of buffer P1. After 5 mins  $350\ \mu\text{l}$  of buffer P2 was added and the samples incubated for 5 mins at room temperature. To this  $350\ \mu\text{l}$  of buffer P3 was added and the samples left on ice for 15 mins. After centrifugation at  $13,000 \times g$  for 15 mins the samples were loaded on the Qiagen column under vacuum, and washed with buffer QC. DNA was eluted with buffer QF ( $500\ \mu\text{l}$ ) at  $50^\circ\text{C}$  and isopropanol precipitated ( $0.8\ \text{vol}$ ). After centrifugation at  $13,000 \times g$  for 30 min, DNA was washed with  $70\%$  (v/v) ethanol and air dried for 10 mins. The final pellet was resuspended in  $10\ \mu\text{l}$  of water. Cycle sequencing reactions using the Perkin-Elmer Applied Biosystems division Big Dye reaction kit (cat No. 4303149) were prepared using standard conditions for plasmid and cosmid sequencing. Samples were analysed on ABI Automated Sequencers. DNA sequences were assembled using the DNA\* software. The complete sequence of cHRIM5 was obtained by primer walking to join the final DNA contigs together. The final sequence of cHRIM5ed2 is shown

in Figure 2. Analysis of the DNA using the software Clone indicated a number of ORF illustrated in Figure 3 and 4. Corresponding protein sequences are also presented at Annex I.

#### Example 7 - Fragments that encode nematocidal activity

To identify smaller fragments that encoded nematocidal activity, a series of sub-cloning experiments were performed using *E. coli* DH5 alpha. Qiagen midi and miniprep methods, restriction and ligations were used as for previous examples. Nematicidal activity of all constructs was determined as described in Example 4. In Figure 4, we show the deletions of cHRIM5 tested for nematocidal activity. Restriction sites and genes are indicated. Size in base pairs indicated on the map line. A cHRIM5, B cHRIM6, C cHRIM7, D cHRIM8, E cHRIM8, F cHRIM10, G *NdeI* deletion of cHRIM8, H Approximate positions (arrows) of three AT2 transposon insertions (tn58, tn26, tn43) in cHRIM9.

The cosmid cHRIM5 (figure 4A) was digested with the enzyme *SaII* and religated. The resulting sub clone cHRIM6, illustrated in Figure 4B showed nematocidal activity. cHRIM6 was digested with the enzyme *SmaI* and religated, producing sub-clone cHRIM7 (Figure 4C). cHRIM7 was digested with *BglII* and the kanamycin resistance gene block (*nptII*, Pharmacia) cut with *BamHI* was ligated into it. After selection on LB containing kanamycin (50µg ml<sup>-1</sup>) and ampicillin (50µg ml<sup>-1</sup>) the clone was digested with *SaII* and religated, in effect creating a deletion from the *SaII* site to the *BglII* site of cHRIM6 to generate cHRIM8 (figure 4D). By cutting cHRIM8 with *NruI* a further deletion was made to create cHRIM10 (figure 4F). All the above clones maintained nematocidal activity.

Deletion of cHRIM8 with *NdeI*, removed a portion of the p14-2f gene (figure

4G), this reduced nematocidal activity. This indicates that the p14-2f gene or protein are important for nematocidal activity. Transposon mutagenesis of cHRIM9 (a clone very similar to cHRIM7 but deleted with *NarI* rather than *SmaI*) with the artificial transposon AT2 (Perkin Elmer Applied Biosystems) resulted in a number of inserts within this clone (figure 4H). Insert cHRIM9-tn43 was restriction mapped to an approximate position of bp 20,700 (on cHRIM5) within the p20-9r gene, this mutant retained nematocidal activity. This indicates that this gene is not essential for activity. Insert cHRIM9-tn58 mapped to an approximate position of bp 13,400 (on cHRIM5), within the p13-1f gene, nematocidal activity was reduced. This indicates that this gene, region of DNA or the blocking effect of the transposon in this position is important for activity. Insert cHRIM9-tn26 was restriction mapped to approximate position of bp 15,000 (on cHRIM5) within the p14-2f gene, nematocidal activity was reduced. This indicates that this gene, region of DNA or the blocking effect of the transposon in this position is important for activity.

Clone cHRIM6-tn43 was digested with *BglII* and *NotI* and cloned into the vector PLEX (Invitrogen cat. No. K450-01) cut with *BamHI* and *NotI*. The *E. coli* strain used was GI742 supplied by Invitrogen. The resulting plasmid insert (PLEX-*BglII*/tn43, Figure 5) places the p14-2f and p13-1f genes under the control of the bacteriophage Lambda  $P_L$  promoter. Figure 5 illustrates the cloning of DNA encoding nematocidal activity in the expression vector PLEX, where: A, plasmid clone; B, insert and gene locations; Tpr, trimethoprim resistance; Apr, ampicillin resistance;  $P_L$ , bacteriophage lambda  $P_L$  promoter; \*, plasmid joins to form a circular molecule; \*\*, incomplete genes. Selection of colonies on RMG media (described in the Invitrogen manual) containing ampicillin ( $50 \mu\text{g ml}^{-1}$ ) and trimethoprim ( $50 \mu\text{g ml}^{-1}$ ) prevents expression from the  $P_L$  promoter. Colonies were then cultured on LB containing Trimethoprim ( $50 \mu\text{g ml}^{-1}$ ) in  $2.0 \text{ cm}^2$  wells for

nematocidal tests. The clone was active. This indicates that genes within this fragment have nematocidal activity. The clone PLEX-*Bgl*II/tn43 was digested with *Cl*aI and religated, this resulted in a deletion of part of the p13-1f gene, this clone had reduced nematocidal activity indicating the importance of this gene.

All these results indicate that the genes and gene products of p13-1f and p14-2f are important for nematocidal activity. Other smaller genes within the *Bgl*II to *Nru*I sites of cHRIM10 and PLEX-*Bgl*II/tn43 may also be essential. In addition genes outside this region within the remaining cosmid clone (cHRIM5) may also encode products with nematocidal activity, or may enhance the nematocidal activity of genes in the smaller region (*Bgl*II-*Nru*I of cHRIM10 and PLEX-*Bgl*II/tn43).

#### Example 8 - Field trials

Activity of strains selected in accordance with the above methods, or from depositary institutions which include bacteria which in nature are associated symbiotically with entomopathogenic nematodes, may be further assessed in field trials as follows.

Symbiotic bacteria in the absence of their nematode host can be inoculated into one or more portions of a field which is infested with nematodes, or into containers containing unsterilised soil from such a field. The bacteria can be inoculated onto the roots of plants, or into seeds. Periodically treated and untreated areas or containers can be assayed for nematode larva, egg, or cyst counts and for the presence of the inoculated bacteria by methods well known to those skilled in the art. A reduction in the number of nematode counts in areas in which the symbiote bacteria are present indicates control of the nematodes otherwise found in the untreated areas or samples.

## Annex I - amino acid sequences

SEQ ID NO:1

P0-0f

ISWFATGIPTVDALLAEFFWHGDKQAFPPFTCRFTHFDPDKEQDVTLPSTEEAYWLHRA  
 LQGQPLHSEVYGDDGTAQAGIPYTVMDSRPQVRLLTGLPGNSPTVWPSVIEQRTWQYERI  
 ADDPQCHQOVVLNSDRYGFPRETVDIAYPRRPKPAVSPYPDTLPATLFDSSYDEQQQQLR  
 LTRQRQHYHHLTDTEHQVLGLPDVMRSDAWGYPAARVPREGFTLEDLLAENSLIAPGTPL  
 TYLGHQRVAYTGTGTGTEEKPTRQALVAYTETAVFDELALQAFNGTLSPEALEKKLIESGY  
 LSVPRPFNTGAESAVWVARQGYTDYGGSEAFYRPLAQRTTVQIGKNTLHWDTHYCAVVRM  
 QDAAGLYTDAAYDYRFLTPVQITDANDNQQHITLTALGQVSSGRFWGTEEGTPQGYTPPE  
 DRPFTTPSSVAEALDLKPDLPVANCVMVYAPLSWMPAHTYQEYIAGFTWQALLDAGVVTE  
 DKRVCALGFRRWVQRQGIVLNGQALADSREP VHVLT LATDRYDTPDQQLRKSVTYS DGF  
 GRLLQSAVYHAPGEAWQRAADGSLITDAKGAPLVAHTATRWAVSGRTEYDGKGQPVRTYP  
 PFFLNAWQYLSDDSARQDLNADTHRYDPLGREYQVRTAKGYLRQNRLTPWFVVNEDENDT  
 LS

SEQ ID NO:2

P1-2r

YLPQRGQCDMLLVVIGIGYLNQGQEAVIDGIRVQTRRILHTDDRTVMGIPMEGVFANLH  
 RRPLSQRTVKRLRPAVIGISLTGDPDRRFRTGIEWAWNRRQITRLD

SEQ ID NO:3

P2-0f

SHLPARYGGRLTTL SRKGFMTVNRGDN LHQKTPEVTVLDNRGLTVRELRYHRHPNTPTTT  
 DERITRHRFTLSGQLAHSIDPRLFDLQQT DNTVNPNIYDTALTGEVVTRTSVDAGNDLI  
 LNDITGRPVLA INATEVTRTWQYENDTLPGRPLSITEQPAGEAGRITERFVWAGNSQAEK  
 NSNLAGQCVRHYDTAGLNQTD SIALNGIPLSVTRQLLPDGT DADWQGNNEPAWNDRLAPE  
 NFTTLSTADATGAVLT TTTDAAGNLQRVAYDVAGLLTGSWLRLAGGTEQVIVKSLTYS AAG  
 QKLREEHGNGVVTTYTYEPETQRLVG IKT KRPQGH AQGTKVLQDLRYEYDPVGNVVKVTN  
 DAEVTRFWRN QKVVPENTYVYDSLYQLVSATGREMANIVQQSTLLPTPSLIDSSTYSNYS  
 RTYNYDRGDNLTQIRHSAPATGNSYTTDITVSDHSNRAVLDTLTDDPAKVDALFTAGGHQ  
 IPLQPGQNLVWTPRGELLKVAPVVRDGOISDQESYRYDAASQRIKTHVQQTANSSQAQS  
 TLYLPGLERHTTINGTTVKEVLHVITIGEAGRAQVRVLHWENGKPGAISNNQMRYSYDNL  
 IGSSGLEVDGDGQIISMEEYYPYGGTAVWTARSQTEADYKTVRYSGKERDATGLYYYYGYR  
 YYQPWAGSWLSADPAGTIDGLNLYRMVRNNEPATLDDKNGLAPGNRYVFFPFIHEDRIFRL  
 ASANVYRTEHNKSDIIA VVEDKALDSKLF TNSIEQFFKKPKGKAILKGS PDIKERLLNNI  
 VHDLSNMQVG DQLYVNAHGHS AKPFFYS DSGYSKIIMEQLQRGANYVAKDLVNKFKLPEN  
 ATIKISTCHSAEGKGAHITVTSTGTNEKMRYSSIIENKGEFSRSLAGTMENELIKLQPGR

39

VRGNVYGYLGATTFFYGAKNEKVIHLKDGNLTTGVHEGKLSMFTKKNRFSENIFGLKVKRS  
LTRTNFTGSGV

SEQ ID NO:4

p-2-9r

PAAEYVRDFTITCSVPPASRSQLPVSRPATSYATRCRLPAASVVVSTAPVASAVLRVVKF  
SGASRSFQAGSLFPCQSASVPSGSSWRVTDSGMPLSAILSVWFSPAVS

SEQ ID NO:5

P3-2r

QRALLNDIGHFAPGGTDQLIQAVIDIGVLRHHFLVAPEAGNLRIVRHFHHVPHRVVLIAQ  
VLQHLRPLCMSLWAFGFYANKALGLRLVGVGGHHAVAVLFAQFLTRGGIRQGFHDNLLCP  
ARKPQPTASQQACYVIRHTLQVTGRIGGGQYRAGGIRRAQGGEVFRCQPVVPGGFIVSLP  
VCVRTIRQQQLARDGQRYAVKRNTVRLVQSGGVIVTHALSGQVAVLLRLTVPCPKTLCDT  
ACFASRLFCDTERASG

SEQ ID NO:6

P3-6r

SDRRQTGYAYSADHYRISGRSTVCTVRAGLMNYQCWLQHAATQLSESDSPKRDAEILLGY  
VTGRSRTYLIADFDETLISSEELHQDLSLLVRRIQGEVPVAYIIGEREFWSLPFAVSPATLI  
PRPDTECLVEKALELLPDSPARILDLTGTGTGAIALALASERNDCYVTGVDINSDAVMLAQ  
HNAEKNAGKLAIHNVNFLQSEWFAAVGNQQQFDMIVSNPPYIDERDPHLQEGDIRFEPATA  
LIAAQNGMADLQAIVGQARHFLSPNGWLLLEHGWKQGTVVRNLFLEKGYQQIATFQDYGG  
NERITIGRWKNKNETHS

SEQ ID NO:7

P3-7f

ARRAVRRCGYCTGRTESRVPSVTTRCATAMITLSAAAVWRWTVTDKLSVWKNTRTGTALR  
CGRRGVRQRLITRLCVTQARSGMQRGCIITATGITSRGRGAG

SEQ ID NO:8

P5-6r

WQNGGSSSTTPRYLAGCYVWYPC SARLSGNAKSLAPDGEWMKHTLKSASGNTFTGRLI  
PTGRPTVVTIDKSGANTAALTLLNAEGEPQQGIEIRQNKYLNNRIEQDHRHVKKRIRPML  
GFKSFRAQT

SEQ ID NO:9



40

P5-7r

ALLFLSESRVMSLIRNAFKLLHYPVDIMAQCVRWSLTIALSLRNLEEMAKRGIFVDHAT  
IPRWVLRLLVPLLSKAFRKRKKPVGSRWRMDETYIKVKGQWKYLYRSVDTDGQTDGCDYR

SEQ ID NO:10

P6-3f

VHSPSGAVAPGKFFIENFADTFPAPLPLHPFIDACIQQGFQLLPCLIAIAHSGKQAFECV  
LLDRALQGSQCLQALVLPVGDVNGQTAHGFLIGYTQTHISTYNGLWLFITQGVRYRFV  
RQTFVCRSLSFSEDDCTN

SEQ ID NO:11

P6-3r

RTCRERPRLMDYVLTKAAEADLRAIIRHTRKQWGDAQVRRYITALEQGIARLAVGQGSFK  
DMSALFPALRMAHCERHYVFCLPRENAPALIVAI FHERMDLLTRLADRLK

SEQ ID NO:12

P6-6r

PQTIICANVGLCITDKEKTMSRLTIDITDRQHQSLKALAALQGKTIKQYALERLFPGMSD  
SDQAWQELKALLDTRINEGMEGKGCGKSIGIILDEELAGSDRA

SEQ ID NO:13

P7-1f

NAHFLIVSKTNVVMNSQDPHNKRDSLFSAPIANLGDWSFDERVAEVFPDMVKRSIPGYSN  
IISMIGMLASRFVTPGSQIYDLGCSLGAATLSIRRSINADNCRIIAIDNSPAMIERCRRH  
IDSFKASTPVEVIEQNILDTDIQNASMVVLNFTLQFLHPDDRQKILKKIYAGLKPGGVLV  
LSEKFN FEDQKIGELLFNMHHDFKRANGYSELEVSQKRSMLENVMRTDSVDTHKSRLKEV  
GFQHVEVWFQCFNFGSLLAIKGTEQ

SEQ ID NO:14

P7-9f

TMIDFGNFYQLIAKHPLNHWLDSLPAQLSHWQKTSQHGOQFSSWVKILENLPEIKPSHLDL  
KNGVIAIHEPDL SKGEKARLHNILKILMPWRKGPFSLYDVEIDTEWRSDWKWERVLPHIS  
PLEGKTVLDVGC GSGYHMRMVGEQAQLVVGIDPTQLFLCQFEAIRKLLGNNQRAHLLPL  
GIEQLPELQAFD TVFSMGVLYHRRSPLDHLWQLKNQLVSDGELVLESLVIEGDENQCLIP  
GERYAQMRNVYFIPSAKMLKVWLEKCGFVDVRIVDHAATTPDEQRRTEWMKTESLVDFLD  
PSDH SKTIEGYAPLRAVLIARKP

41

SEQ ID NO:15

P8-4r

SLQIDREKVGLD RYPQPIERLRQPCATCDNHCHSRHQVRFFLLKEKYGAALAPISSQSAI  
RYQFQRHTMKKGLFAMASIFSGYCGGELFHLLTDPAHESQ

SEQ ID NO:16

P9-8r

SSFRLNDDLLTNSYSEGFLMIKLEICCYSSISCALVAQNAGADRIELSASPLEGGLTSPSFG  
ALQQSLQRLSIPVHPIVRPRGGDFCYNMDFEAMKNDVARIRDMGFPGIVFGILSENGHI  
DRLRMRQLMSLSGNMAVTFHRAFD MCFNPHVALEQLTELGVQRILTSGQQQNAELGLTLL  
KELMQASRGPIIMPGAGVRVSNISKFLEAGMTEVHSSAGKIVPSTMKYRKVGVMSSDDR  
DVDEYSHYSVDGELVESMKGVMSLIKR

SEQ ID NO:17

P10-5r

YFGKNRRFVIYVTLMERNFYGLFNGEEMSHFSKISELQDLVADLAGFEQKLKQFEGHLGL  
HFEQYSADHISLR CNESKIADRWRKGFLQCGQLISESIINGRPICLFDLNQPIVLLDWKI  
DCVELPYPSQKHVYHQWEHVELVLPVPPEQLICEAKKLLPQPLPDNFRMKESH PKGKNE  
RLPNPILAV

SEQ ID NO:18

P10-7f

GNTVNIQVILSEKISNALIEAGAPT DSEAHVRQSAKAQFGDYQANGVMAAAKKVGIPPRQ  
LAEKVVSQDLQGIASKVEIAGPGFINIFLDKAWVAANIETTLKDEKLGITPVEPQTIVI  
DYSAPNVAQMHVGH LRSTIIGDAAARTLEFLGHKVIRANHVGDWGTQFGMLIAYLEKIQ  
NENANDMALADLEAFYREAKKH YDEDEEF AIRARNYVVKLQGGDEYCRKMWRKLVDITMS  
QNQETYNRLNVTLTEKDVMGESLYNDMLPGIVADLKQRGIAVKSDGATVVYLDEFKNKEG  
EPMGVIIQKKDGGYLYTTTTDIACAKYRHETLNASRVLYYIDSRQH QHLMQAWAIVRKTGY  
IPESMSLEHHMF GMMLGKDGKPFKTRAGGTVRLSDLLDEAIERADTLIREKNPDMPEDEL  
KKVVEAVGIGAVKYADLSKSRTTDYVFDWDM LAFEGNTAPYMQYAYTRVSSIFKRADID  
ENSLTLPVMLNEEREQALATRL LQFEETITTVAREGTPHVMCAYLYDLAGLFSGFYEHCP  
ILNADSEELRQSR LKLALLTAKTLKQGLDTLGIQTVERM

SEQ ID NO:19

P11-1r

AQVSNMHLLGDIRCGI IDNDGLRFHWGDTELFIFQGSFYICCNPRFIKKNIDKTWACNFN  
FAGNSLQIQ LADDFFCQLSRRYSHLFSGSHHTIRLIVTKLCFGR LTDVSFTVGWSASFNQ  
RIADFF

SEQ ID NO:20

P12-1r

HARVGVLHIRCRVAFKGQHIIPVENIVCSTALGKICIFHRANPYRFHDFQFVFWHIWVF  
LTNEGIRTLNRFIQIGQSYCAAGTGFEWFTIFAQHHAKHVFE

SEQ ID NO:21

P12-5r

YHASFQLCRLLHTFYSLNTQSIKTLQSFRCQQSQLQAALAQFFAIGIQDRAVLIIETRE  
QTGQIVQVCTHNMWRTFTGDGSDRFFKLQQAGCQCLLAFFIQHHRQCQAVFIDIRTFKDR

SEQ ID NO:22

P13-1f

FTLREDSMSDWTGVSTFNVILETGLDNCNIYANGLNMIGVIINITPTDDEGNFVDIDDDVT  
LNDNIKIVDYIDGSDIDGSDGWFTGNPNEYNTIPNSQSYSLKSENSQITQIKRYVSCS  
NTSRLRTKSFSKAVTTTSGKVISITQNSINSSRVVINAIDATNFTDDELRTTKETRFENQ  
SYTSHKSSTNSLYVHTWTIPRSLKLQNRWEDYNNGWTWAQSCYYKTGADGGSESTRWLA  
AGSIFPPGNYDGLWLDNDIALSGMAHKSYNVDTGINQLSFTRIIGKGFVWVYNISGLDRG  
HAVIIIDQYGNKYRILFHAGYENS DPYLSSSIVY

SEQ ID NO:23

P14-2f

VYIKFLKLFRRITMSDNNEFFTQANNFTSAVSGGVDPRTGLYNIQITLGHIVGNGNLGPT  
LPLTLSYSPLNKTDIGFGIGNFGLSVYDRKNSLLSLSTGENYKVIETDKTVKLQKKLD  
NLRFEKDLKENCYRIIHKSGDIEVLTFGNNAFDLKVPKLLNPAGHAIYIDWNFEATQP  
RLNRIYDDLDGHDIPLLNLEYQGLIKTILTLFPGQKEGYRTELRLNRQLNSIHNFSLGN  
ENPLTWSFGYTPIGKNGILGQWITSMTAPGGLKETVNYSSNNQGHHPQSANLPVLPYVT  
LMKQVPGAGQPAIQAEYSYSHNYVGGGSGNGIWNKLDNLYGLMTEYNYGSTESRRYKDK  
EGHDQIVRIERTYNNYHLLTSECKQONGYIQTETAYYAIIGHNFDSQPSQFQLPKTKTE  
TWRSADNSYRSEITETTFDESGNPLTKVIKDKKTQKIISPSTHWEYYPAGEVDNCPPEP  
YGFTRFVKKI IQTPYDSEFKDDPEKFIQYRSLIGSQSHVTLKIEERHYSATQLLNSTLF  
QYNTDKSELGRLLKQTECTKGENGKTYSVVHKFTYTKQDDTLQQSHSITTHDNFTIHRSQ  
VRSRYTGRLFSDDTKDIVTQMSYDKLGRLLTRTLNSGTPYANTLTIDYELNNLQDDNRP  
PFVITTTDVNGNQLRNEFDGAGRHSVQCLKDSGDGKFYTIHTQQYDEQGRHHTSTYSYD  
LTNGRQQTD PDKVHLSMSKSYDNWGQIANTHWSYGVSEKITVDPITLTATKQLQSNSNNV  
QTGKEVTTYTPSQPIQITLFDEAGHLQSCHTLTRDGWDRVRKETDAIGQCTIYQYDNYN  
RVIQITLPDGTIVNRKYAPFSTDTLITDIRVNGISLGQOTFDGLSRLTQSQDGGRVWAYT  
YSAGNDQCPSTVITPDGQFIHYQYQPELDDAVLQVASNEITQQFSYNPVTGALLKAVAEG  
QSLTPIIYPSGRLKMNINDMKKMSYLWTLRGLENGYD L TGTIQKISRDT HGRVTQIKD

43

SSIKTTLNYYDDLNRHIGSQVTDLATGHMLTTTVEFDGLNREIGRKLCDSSGHTLDIQQSW  
LKTQQLANRIVKLNGLVLRTEQYSYDSRNRLNQYKCDGAECPTDKYGH SIVTQNFTYDIY  
GNITACHTTFADGTEDHATFKFANPTDPCQLTEVHHTHPDMFDNIRLKYDKAGRVINITD  
NHGNTENFTYDTLGRQLQNGQGSVYGYDPLNRLVVSQKTDTLDCELYRETMLVNEVRNGEM  
IRLLRTGETIIAQQRASKVLLTGTDSQQSVILTSQKQNLSEAYSAYGKHKSTANDASIL  
GYNGERADPVSGVTHLNGYRSYDPTLMRFHTPDLSLSPFGAGGINPYSYCLGDPINRSDP  
SGHLSWQAWTGIGMGIAGLLLTATGGMAIAAAGGIAAAIASTSTTALAFGALSVTSDIT  
SIVSGALEDASPKASSILGWVSMGMGAAGLAESAIAKGGTKLATHLGAFADGENALLKST  
SESSRIKWGVTRSLDREIVRNEEGQVIKDHSGYTDNFMGKGEQAILVHGDKDGFYHTE  
GNKHNGKGPYTRHTPEQLVDYLDNNIVDLTQGGDKPVHLLSCYKSSGAADKMAKYINR  
PVIAYS NKPTISQGLARIERKDFFLKSTYHSYDPRKIILGRTEKTVKPKTFRP

SEQ ID NO:24

P17-6r

LCYGHICLSGIPHRHIYIGSTYYGNRKSTVLYAAILHSVSLFYLLIAVFSASSAGYLTYG  
LSYHTISVQFLGLSHQIPLLLSTYDQSLNLLLDYQYGDSGHRNLE

SEQ ID NO:25

P17-8r

SAQCIVGKVFRISMVISDIYYSTSLIIFQPDIIIRHIWMSVVYLCQLAWVSWVGKFECSMV  
FCPICECGVTGGDIAIDIISKILCDYAMAI FVCRAFRTVTFILVQPITGIVRVLFCTLQY  
SIQFHYSIC

SEQ ID NO:26

P18-7r

PSSLRTISLSKLLVTPHFILELSEVDLSKAFSPSSANAPRCVASLVPPLMADSANPAAPI  
PIETHPSIEDAFGEASSAPLTIDVISDVTLAPNASAVVEVEAIAAAIPAAAIAIPPV  
AMVSSNPAIPMPIPVHACQLK

SEQ ID NO:27

P19-5f

AHCHIALFPCWHNPQYCQQHPDHHSNCHHQFKQEYPPSRQRRENITLTQLPIKHTGIEAG  
SQTNRKRQTCMFQRANESKVHQLGQNGQRDRNFYWCFDILT

SEQ ID NO:28

P19-8f

PQSTPSSQNSRQLTPAESSQHQQKQSDHIEIMIPSEAPREYREQLHKATPARNRDVAPNP  
SVFDILRDYHWKNFSPVKAAKSSLTPHPVHQKAIPLNDQRNTSMKQSLKPEMRQKLY

SEQ ID NO:29

P20-1r

GKNCINDQGNLPDRYTQNCRPHLTDNPPYGTVERTNPRQYQHADLFQMRKLIGQLQNPSC  
NNGPTQRQHWRIAIRSHKQCKNDHTDIEQCRSKSRHRKAVPCIKNCASQRSQRNQKDIRK  
RNSK

SEQ ID NO:30

P20-9r

NNTMNLKSLAAVSSMTMFSRVLGFIRDAlIARIFGAGMATDAFFVAFKLPNLLRRIFAE  
GAFSQAFVPILA EYKNQQGDEATRTFIAYISGMLTLILAIVSVIGVIAAPWIIYVTAPGF  
TDTDPKFVLT RDLLRITFPYIFLISLASLAGAILNTWNRFSVPAPAFPTLLNVSMIIFALF  
VAPYCNPPVLALGWAVVAGGVLQLAYQLPHLKKIGMLVLPRI SFRDSAVWRVIRQMGP AI  
LGVS VGQISLIINTIFASFLVSGSVSWMYADRLMELPSGVLGVALGTILLPSLAKSFSS  
GNHEEYRK LMDWGLRLCFL LALPCAVALGILAEPLTVSLFQYGHFSAFDAEMTQRALIA Y  
CFGLMGLIVVKVLAPGFYSRQDIKTPVKIAIATLILTQLMNLAFVGPLKHAGLALSIGLA  
ACFNASMLYWLKRKDI FTPLAGWGIFL FKL VVAIAVMVGVL LAVLWVMPAWEQGNMAMR  
LLRLMGVVIAGAGSYFAVLALMGFRLKDFAHRLQ

SEQ ID NO:31

P21-7r

AIILIRDKLSRIFSRQISGEGMFGYRSASPKIRFITDRMVVRLVYERDAYRLAEYSENK  
DFLKPWEPT RDGSFYQPSGWTNRLNYIAELQRQ NATFN FVLLDSDEREIMGVANFTNVVR  
GAFHSCYLGYSLAEKLQGGQGLMYEALQPAIRYMQR YQRMHRIMANYMPHNHRSGNLLKKL  
GFEQEGYAKNYLMIDGVWQDHVLTALTDDAWGKVGL

SEQ ID NO:32

P21-8f

WCAMSLVSQARSLGKYFLLFDNLLVVLGFFVVFPLISIRFVEQLGWAALIVGFALGLRQL  
VQQGLGIFGGAIADRF GAKPMIVTGMLLRALGFALMAMAHEPWILLSCVLSGLGGTLFD  
PPRAALVIKLRPHERGRFYSILMMQDSAGAVVGALIGSWLLQYDFNIVCWIGASIFVLA  
ALFNAWLLPAYRISTIRTPIKEGMMRVIRD RRFlyyVLT LTGYFVLSVQVMLMFPIIHE  
ITGTPTAVKWMYAIETAISLTLLYPIARWSEKHFRLEQRLMAGLFLMSICMFPIGWVNQL  
HTLFGLLCLFYLG LVTADPARETLSASLSDPRARGSYMGF SRLGLALGGAIGYTGGGWLY  
DTGRDLNMPQLPWILLGLSGLITIYALHRQFNQKKIDPVMLGRH

SEQ ID NO:33

P23-1f

45

KGANMKRFFLGAALVLVGLVSGCDQFKDFSINEGLMNDYLLKKVHYQKKISIPGIANANI  
TLGDLSSQIGRQDPEKIELSTQAKVQLATLLGTIQADMKLTIKAKPVFDAEKGAIFVKGL  
EIVDYQTTPEKAAAPVKALIPYLNTSLSEFFDTHPVYVLNPEKSKAEAAAASQFAKRLEIK  
PGKLVIGLTDK

SEQ ID NO:34

P24-4r

QVALQHGRRLGTITLFDNLLGLNQVMNEFSIVCRILGTLENRAPQDPVLQPLITMIAEGK  
LKQAWPLEQDEWLDRLQQNSELSVMAADYHALFTGESASVAVCRSDYTDGEESEVRQFLT  
ERGMPLSDTPADQFGSLLLAVSWLEDQAAEDEIQAQITLFDEYLLPWCGQFLGKVEAHAT  
SGFYRTLAIVTREALQALRDELESE

SEQ ID NO:35

P25-3r

DCMNIIFFHPSFNTDEWIIQGIQARLPDAKVRQWVSGDQEPADYALVWQPPYEMLANRQGL  
KGIFALGAGVD AIFKQESKNPGTLLADVPLIRLEDTGMGRQMQEYAITSVLHYFRMDEY  
KRYQEQRLWNPIAPHNRKEFVIGVLGAGILGRSVIGKLMEFDNVRCSRTSKQLDSVES  
FYGKEQLGDFLSGCKVLINLLPDPDTRGILNLSLFSQLKSGSYVINLARGAQLVEQDLL  
VAIDKGYIAGATLDVFAEEPLSNMHPFWTHPRINVTPHIAANTIPEAAMDVICENIRRMV  
QGEMPTGLVDRVRGY

SEQ ID NO:36

P26-0f

KTSQGFTSTTCSNGNVLKICGLITPCSSLIQRTYPNNMTIGIFSKESTAKNFGMGFLYYF  
DLRVLSPFFKAPINIFTGWQHNTNFRKSRNSTIRLCSSTPNSKQYFTTSRKCHITGAGKY  
RFSIENCFIKSG

SEQ ID NO:37

P27-0r

YSAGCSTVLKSSLNLQCDTFNCESFVMLTLNFTSVNAKPSHIWAHYVDFDLRKKWEVDL  
EYFQFEGEVKTGOYGRMILSGMPEIRFYLSNIEVNKEFTDQVNLPMGILTRHQIITDE  
NNMACRVQVTVSFEPDANIPAVQAESFFKQGTQDLVESVLRKSVVETVSPKPNLQLVYV  
SDIESSTAFYKTI FNAEPI FASSRYVAFFPAGGEVLFAIWSGGAKPDRAIPRSEIGIMLP  
SGKDVDRCFEEWRKNPEIKIVQEPHTEVFGRTFLAEDPDGHIIRVCPLD

SEQ ID NO:38

P27-8r

KGNQITMILYKGSKNYLFNQLNYDSCVLLLEVDES VN LNGWDELSRAQRLLFLMEILRRYH

46

FPVQGVLAQKLNISLRTLYRDIASLQAQGAIIEGEPGIGYVLRPGFVLPPLMFTQNEIE  
ALALGANWVAKRADPQLKESANNAISKIAAVIPAELKQMLEASSLLIGPAATAVQPVVEI  
QQIRQAINTRHKITLAYLDIKDIPSERTIWPFFALGYFENISIVIGWCELREEFRHFRSDR  
IMRLKIENQCYPRSRQVLLKEWRAMEKISR

SEQ ID NO:39

P27-9f

RKMTIYDLKPRFQNLRLPIVIYLYKQGITANQVTLTALFLSIFAGSLLSLFSPHLYWLL  
PVFLFIRMALNAIDGMLAREHNQKSHLGAIYNELGDVISDVALYLPFCLLPDVNSLSLLI  
ILFLTILTEFIGVLAQTIGASRRYDGPIGKSDRAFI FGAYGLIIAIFPLALGWSISLFAF  
MIILLVTCYQRVVKALREIRLAEQSHSK

SEQ ID NO:40

P28-5f

GVNMTPQLDQRIAEHYFTTSDNASLFYRYWPQQQANPDRAIIIFHRGHEHSGRIQHVV  
GLDLPDVPMFAWDARGHGKTEGPRGYSPSMGTSIRDVDEFVRFIATQYGIAMENIVVIGQ  
SVGAVLVSAVVDYAPKIRAMILAAPAFDIKLYIPFATQGLQLMQKARGIFFVNSYVKAR  
YLTHDETRIASYNSDPLITREIAVNILLDLYQTAERVVKDAAITLPTLLFISGSDYVVN  
KKPQHGFYQQLNTPIKEKHVMDGFYHDTLGEKDRHLVFDKIRVFIERIFALPRYQHDYSQ  
EDTWSHSADEFRTLSTSLPCLCPKKLSYQLMRKVMSTHWGRTSEGVCIGLKTGFDSGSTL  
DYVYRNQPPQKGILGRILDKHYLNSIGWRGIRQRKIHIEMLRHAIRSLREQNMPVHMVD  
IAAGHGRYILDAINDFSKVDSILLRDYSEINVNQQAIEERDLTDKIRFIIGDAFNAES  
ISSITPAPTGLIVSGLYELFPDNNLLRNSLRGFADVMTENGYLVYTGQPWHPQIEVIARV  
LSSHRDSQPWIMRRRTQGEMDALVEAAGFEKLYQLTDNWGIFTVSIKRVHR

SEQ ID NO:41

P28-5bf

HHNSINVLLKNIISP HQIMLLCFTVTGHNNRPIQTERSLLFTVVMSTQDVSSMSLTDSIC  
LMFLCSRGMPPVDTVRQKGRAVTAHPWERRFVMLMNLSDLLPLSTASPWKISWLSARVSE  
Y

SEQ ID NO:42

P30-3f

INKYKMEHHMHSSLDSSRRRLWLTGVIWLLFLAPFFFLTYGQVNQFTAQRSDVGTVMFGWE  
HNIPFWSWSIIPYWSIDLFGYISLFICTHRREQWLHGWRLMTASLIACVGFLLEPLKFSF  
SRPTTEGLFGWLFNQLELFDLPYNQAPSLHIILLWLLWLRYSAYVSGYWRGLLHIWSVLI  
ALSVLTTWQHFFIDVLTGFAVGVILSYLLPVSYRWRWQPNQDRYARKLFGYYLTGSALFA  
LIASLLGGFSWILLWPAVSLLMIALGYAGLGSSVFQKQPDGRMSLSARWLLAPYQLGAWL  
SYLWFRRKSAFPNHITEGIIIGSLPCQPVTAVSVLDITAEWHRRSDARTVNYVCQPQIDL

47

LPLAPEALQSAVCTLDKLRQQGDV FVHCTLGLSR SAMVVAWLLKQHPEYDINTV VAILR  
KARPHVTFRQTHLDALSQWAKGYL

SEQ ID NO:43

P31-6f

QSCVKPDRMSRSDKHIWMPCLNGQKATYNGEHNMQPENLISKVIIATLKSWRFISTLSAF  
SILIATAMLI AVFNTTALNNIALYAVLLFTTLYCQYYCWRTWLDCHYFQILNSSPEKSAE  
FDQTLILLIFNKLPQSRTQNDRENGAIKLLKKATIGLILQWILFFLFLTLKYSA

SEQ ID NO:44

P32-3f

MNTRKINGIRPFSAFIDSCLESYSFPRFIRDIIAGITVGVIAPLAMALAIGSGVAPQY  
GLYTAAIAGIVIAMTGGSRYSVSGPTAAFVVILYPVSQQFGLSGLLIATLMSGVILIVMG  
LARFGR LIEYIPMSVTLGFTSGIAIT IATMQVQNF FGLKLAHIPENYIDKVVALYQALPS  
LQLSDTLIGLTTLLVLIFWP KLGVKLP GHLPALIAGTAVMGAMHLLNHDVATIGSSFSYT  
LADGTQGQGIPPILPQFVLPWNLPDTHSLDISWNTVSALLPAAFSMAMLGAIESLLCAVI  
LDGMTGKKHHSNGELLGQGLGNIAAPFFGGITATAAIARSAANVRAGATSPIAAVVHSL  
VLLTLLVLAPMLSYP LAAMSAILLIVAWNMSEAHKVVDLIRHAPKDDIIVMLLCLSLTV  
LFDMVRRDHYRHCAGITPVYAQNCQYDSNQHVIFNKRGERVIGRTN

SEQ ID NO:45

P33-4r

ESIGAKTSNVNNTSRECTTAAIGEVAPARTLAAERAIAAVAVMPPKKGAAILPNPWPSSS  
PLEWCFFFPVIPSRTAHSNDSIAPSMAIENAAGSNADTVFQLISRECVSGKFHGRTNWGR  
MGGMP

SEQ ID NO:46

P33-5f

LSYSIWSVAITIGIVLASLLFMRKIANMTRISTSSLTSAEKGLLVVRINGPLFFAAAERI  
FAELREKSADYQTIIMQWDAPVLDAGGLHAFQGFVRELGKEKHIVVCDIPFQPLKTLAR  
AKVMPIEGELSFYATLPKALKEMAVDYTPEVCASSEKIQQQ

SEQ ID NO:47

P34-3f

CMSDVENDRRTLGSLLDTEAQHVNHQIVITKVAATVTQDHLVIAAFFEFFNNIAHL PRA  
NKLWFFNINHSTGFRHRFNQIGLAGKEGWKLNHIHHIRDWLSLCRLMHVSDNFHAEGLFQ  
FLKDFHPLFQPWPTIRDRRTVSLIKRRFKNIRNAQFLCHGDIVLTNPHGQIP



SEQ ID NO:48

P35-0r

LSCIRFIFLLIQIYLPLTREGISMQQKVVNIGDIKVANDLPFVLFGGMNVLERSDLAMR  
ICEHYVTVTQKLGIPYVFKASFDKANRSSIRSRYRGPGLLEGMKIFQELKQTFGVKIITDV  
HEPAQAQPVADVVDVIQLPAFLARQTDLVEAMAKTGAVINVKKPQFVSPGQMGNIVEKFK  
EGGNDQVILCDRGSNFGYDNLVVDMLGFGVMQQATQGAPVIFDVTHALQCRDPLGAASGG  
RRAQVAELARAGMAVGIAGLFLEAHPDPENAKCDGPSALPLAKLESFLMQIKAIDDVVKN  
FPELDTSK

SEQ ID NO:49

P35-8r

VDGIKMKPIVNYEFNNTPLIDGIILVSKIIRPDFPQTLVSEQLTALVEEARQRLSSITDS  
KVKLDSLLTLFYREWKFEGGANGVYCLSDTLWLDRLLSRQSPVSLGTVFTHIAQALGLS  
VQPVIFPIQLILRIDLLDQPTWFINPLNGDTLNEHTLDVWLKGNIGPTVRLKKQDLQEAD  
NVSLVRKITDTIKVSLMEEKKMELALKASEVVLTFDPPDPYEIRDRGLIYAQLDCNHIAV  
SDLSYFVEHCPEDPISEMIKMQINTIEQRLIVLH

SEQ ID NO:50

P36-7r

SDRRQTGYAYSADHYRISGRSTVCTVRAGLMNYQCWLQHAATQLSESDSPKRDAEILLGY  
VTGRSRTYLIADFETLISSEELHQLDSSLVRRIQGEVAYIIGEREFWSLPFAVSPATLI  
PRPDTECLVEKALELLPDSPARILDGTGTGAIALALASERNDCYVTGVDINSDAVMLAQ  
HNAEKNAGKLAIHNVNFLQSEWFAAVGNQQFDMIVSNPPYIDERDPHLQEGDIRFEPATA  
LIAAQNGMADLQAIVGQARHFLSPNGWLLLEHGWKQGTVVRNLFLEKGYQQIATFQDYGG  
NERITIGRWKNKETHS

SEQ ID NO:51

P37-5r

VEMREMAQEELKEAKIRNEELEQQQLQLLLLPKDPDDERNCFLEVRAGTGGDEAAIFAGDL  
FRMYSRYAEARRWRVEIISANEGEHGGYKEVIAKVSGDQVYGHCLKFESGGHRVQRPETE  
SQGRIHTSACTVAVMPEIPEAELPDISPGDLKIDTFRSSGAGGQHVNTTDSAIRITHLPT  
GIVVECQDERSQHKNAKAMSVLAARIRAAEMRKRQEVEASERRNLLGSGDRSDRNRTYN  
FPQGRVTDHRINLTLYRLDEVIEGKLDMLIQPIIIIEYQADQLSALSEQD

## Claims:

1. The use of a bacterial strain to control a target nematode, characterised in that in nature the bacterial strain is associated symbiotically with an entomopathogenic nematode.
2. The use according to claim 1, wherein the bacterial strain from nature is directly employed to control the nematode target, or is employed to give a recombinant bacterium employed to control the nematode target, or the natural or recombinant strain is employed as a source of a nematode control agent to control the nematode target.
3. The use according to claim 1 or 2, wherein the target nematode is not the same as the nematode with which the bacterial strain is found symbiotically in nature.
4. The use according to claim 1, 2 or 3, for control of helminthiasis in a human or a domesticated animal or the control of plant pathogen nematodes.
5. The use according to any preceding claim wherein the nematode to be controlled comprises one or more of *Haemonchus*, *Trichostrongylus*, *Ostertagia*, *Nematodirus*, *Cooperia*, *Ascaris*, *Bunostomum*, *Oesophagosromuni*, *Chabertia*, *Trichuris*, *Strongylus*, *Trichonema*, *Dictyocaulus*, *Capillaria*, *Heterkis*, *Toxocara*, *Ascaridia*, *Oxyuris*, *Ancylostoma*, *Uncinaria*, *Toxascaris*, *Parascaris*, *Aphelenochoides*, *Anguina*, *Bursaphalenchus*, *Criconemella*, *Melodigyne*, *Ditylenchus*, *Globodera*, *Heliocotylenchus*, *Heterodera*, *Pratylenchus*, *Radopholus*, *Rotelynychus*, *Tylenchus*, *Trichodorus*, *Xiphenema*, and *Caenorhabditis*.

6. A composition for the control of parasitic nematodes which comprises as an effective agent a species of bacterium which is a symbiont of an entomopathogenic nematode, or an engineered bacterium, or a nematode control agent derived from a natural or engineered bacterium.
7. A composition according to claim 6, wherein the bacterial species is of the genera *Xenorhabdus* or *Photorhabdus*,
8. A composition according to claim 7, wherein the bacterial species is of the genus *Xenorhabdus*
9. A composition according to claim 8, wherein the bacterial species is of , the species *Xenorhabdus bovienii*.
10. A composition according to claim 8, wherein the bacterial species is:  
*Xenorhabdus bovienii* strain H31 deposited with NCIMB under accession number NCIMB 40985;  
*Xenorhabdus bovienii* strain I73 deposited with NCIMB under accession number NCIMB 40986; and  
*Xenorhabdus* strain C42 deposited with NCIMB under accession number NCIMB 41004.
11. A composition according to any of claim 6, wherein the nematode control agent which is derived from a symbiont of an entomopathogenic nematode or from an engineered bacterium has functional activity against a nematode, and is a peptide.
12. A nucleic acid encoding a peptide of claim 11.
13. A nucleic acid according to claim 12, which nucleic acid comprises a

natural nucleotide sequence or a degeneratively equivalent sequence, or a functional variant thereof.

14. A nucleic acid according to claim 13, which is a homologous variant encoding a peptide which is a nematode control agent, the nucleic acid having 70% or more DNA sequence identity and/or the peptide having 70% or more amino acid sequence identity.
15. A nucleic acid according to claim 13, which is all or part of cosmid cHRIM5, in particular p 13-1f or p 14-2f, and variants thereof.
16. A nucleic acid according to claim 13, 14 or 15, wherein the variant has a sequence which is a derivative by way of addition, insertion, deletion or substitution of one or more nucleotides.
17. A nucleic acid according to any of claims 12 to 16, which is part of a longer sequence and the nematode control agent is expressed as a fusion protein.
18. A nucleic acid complementary to a nucleic acid according to any of claims 12 to 17.
19. A nucleic acid for use as a probe or primer having a nucleotide sequence of at least 15 nucleotides, which sequence is present in a nucleic acid according to any of claims 12 to 18.
20. A method for identifying or cloning a nucleic acid according to any of claim 12 for a nematode control agent, which method employs a nucleic acid probe according to claim 19.

21. A method according to claim 20, which comprises the steps of:
- (a) providing a preparation of nucleic acid from a bacterium,
  - (b) providing a probe,
  - (c) contacting nucleic acid in said preparation with said probe under conditions for hybridisation of probe to any said gene or homologue in said preparation, and,
  - (d) identifying said gene or homologue if present by its hybridisation with said probe.
22. A method according to claim 20, which comprises the use of two primers to amplify a nucleic acid encoding a nematode control agent, at least one of the primers having a conserved nucleotide sequence of at least 15 nucleotides.
23. A method according to claim 20, which comprising the steps of:
- (a) providing a preparation of nucleic acid from a bacterium,
  - (b) providing a pair of nucleic acid molecule primers, at least one of which is a primer,
  - (c) contacting nucleic acid in said preparation with said primers under conditions for performance of PCR,
  - (d) performing PCR and determining the presence of absence of an amplified PCR product.
24. A recombinant vector comprising a nucleic acid according to any of claims 12 to 17.
25. A host cell containing a vector according to claim 24 capable of replication.
26. A host cell according to claim 25 which is a plant cell.

27. A method for producing a transgenic plant which comprises the step of regenerating a plant from a plant cell according to claim 26.
28. A plant produced according to claim 27 which is a crop species which can be maize, cotton, soya, rice, *Brassica* species, tomato, potato, sugar beet, barley, soybean, peanut, onion, rye, wheat, corn, banana, raspberry, bean, or a decorative or other plant.
29. A method of producing a peptide nematode control agent comprising causing or allowing expression of a nucleic acid according to claim 12.
30. An antibody or fragment thereof, or a polypeptide comprising the antigen-binding domain of the antibody, capable of specifically binding a peptide of claim 11.

1/14

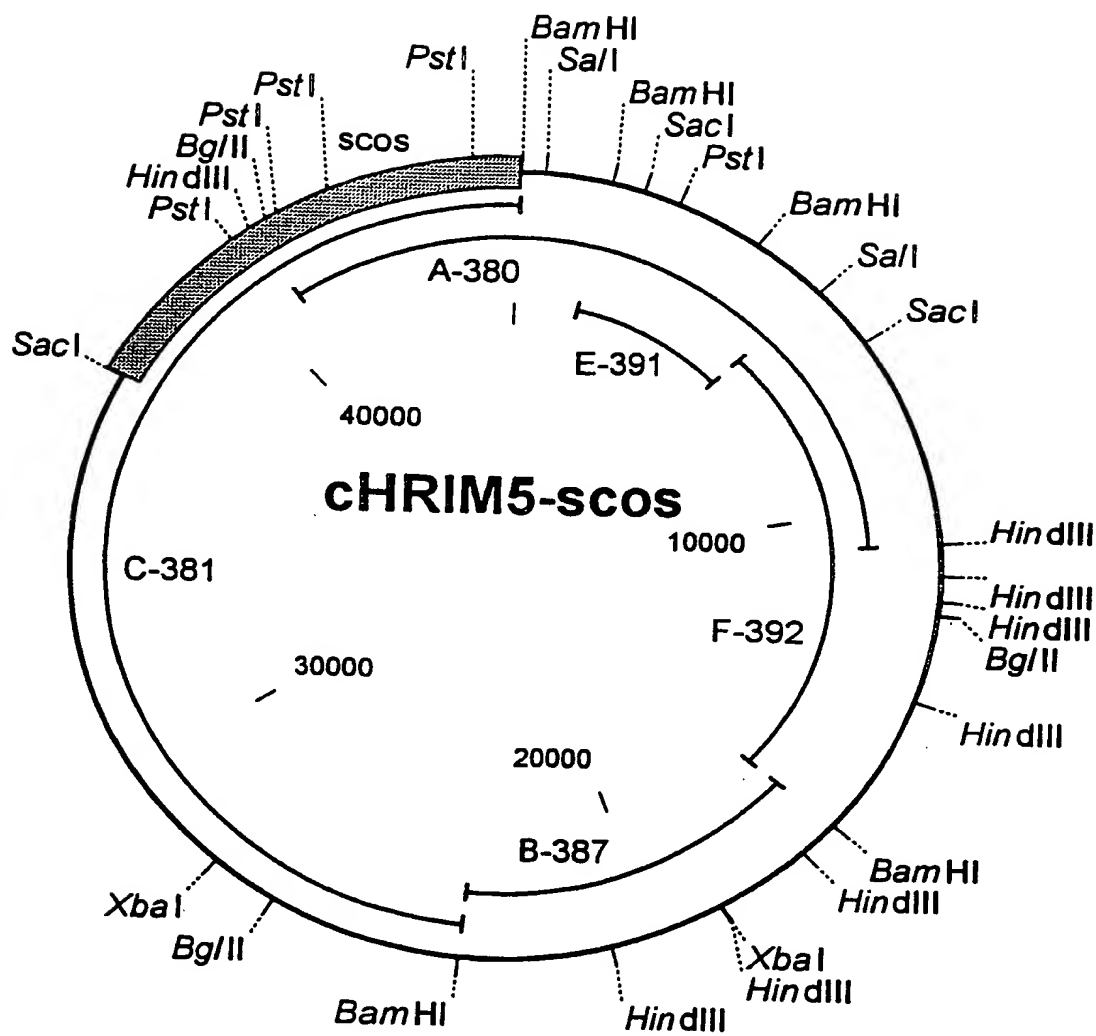


Fig. 1

2/14

Molecule:

Sequence Data

Description: chrim5ed2.seq, 37544 bps DNA

1	ggatcagctg	gttttgccacc	gggatcccca	ccgttgatgc	cctgttagcg	gaggaattct
61	ggcacggtga	caaacaggct	ttcccgccct	ttacctgccg	ttttacgcat	tttgaccctg
121	ataaagaaca	ggatgttact	ctcgttcctt	cgacggaaga	ggcttattgg	ctgcaccggg
181	cgttgcaagg	ccaaccgtta	cacagtggag	tctatggcga	cgatggcacc	gcgcaggcgg
241	gtatccccta	taccgttatg	gacagtcggc	cccaggttcg	gcttctgacg	ggtttacccg
301	gtaactcacc	gacagtctgg	ccgagtgtga	ttgaacagag	aacctggcag	tacgaacgga
361	ttgccgatga	tccgcaatgc	catcagcagg	tgggtgctgaa	cagtgaccgc	tacggttttc
421	cacgggagac	cgtcgacatt	gcttatccgc	gccgccctaa	gcctgcggtg	tcaccttacc
481	cggatacgtc	gccggcgacg	ttatttcgaca	gcagctatga	tgagcagcaa	cagcaattgc
541	ggcttaccgc	gcaacggcaa	cattaccatc	acctgactga	cactgaacat	caagtgtctg
601	gactgcctga	tgtcatgcga	agcgtatgct	ggggctatcc	ggcagcgcg	gtaccccggtg
661	aagggtttcac	cctggaggac	ttgctggcag	agaacagtct	gatagccccc	ggcagcccat
721	tgacctattt	agggcatcaa	cgcggtggctt	ataccggaac	gaccggaacc	gaagaaaaac
781	cgacccgaca	ggcgctggtg	gcttataaccg	aaaccgcggt	ttttgatgaa	ttggccttgc
841	aggccttttaa	tggcacattg	agtcctgaag	ccctggaaaa	gaaattaatc	gagtctggtt
901	atttgtctgt	tccacgccc	ttcaataaccg	gtgcggaatc	ggcggctctg	gtcgcctctc
961	agggatatac	cgattacggc	gggtctgagg	cgttttaccg	tccgttggct	cagcggacga
1021	cggtgcagat	tggcaaaaac	accctccatt	gggataccca	ttactgtgcg	gtcgtccgta
1081	tgcaggatgc	ggcgggtctg	tacacggatg	ccgcctatga	ttaccgcttc	ctgacccccg
1141	ttcagataac	cgatgccaat	gacaaccagc	aacatatcac	actgaccgcg	ctggggcagg
1201	tatcatccgg	ccggttctgg	ggcactgagg	aagggaactcc	gcagcgttat	atcccgctgtg
1261	aagaccgccc	atttacgcca	ccgtcctcag	tggcggaagc	cctcgacttg	aaaccggatc
1321	ttccggttgc	caactgcatg	gtttatgcgc	cgctgagttg	gatgccgttg	gcgcacacct
1381	atcaggaata	tatagccggc	tttacgtggc	aggcactgct	tgacgcgggg	gtagtgcagg
1441	aagataagcg	ggtttgtgcg	ctgggtttcc	gtcgctgggt	gcaacgtcag	gcatttgtgc
1501	tgaatgggca	ggcattggcc	gattcacggg	aaccgcgtcc	tgtcctaacg	ctggccactg
1561	accgttatga	cacggatccc	gatcagcaac	tgcgcaagag	cgtcacctac	agcgacggct
1621	tcgggcgttt	attgcaaaag	gcagtctacc	atgcgccagg	agaagcctgg	caacgcgcgg
1681	catagggcag	cctgatcacg	gacgcgaaag	gggcgcccct	cgtagcccat	acggcaaccc
1741	gctgggcggg	ctcaggcagg	acagagtatg	acggtaaaag	gcaaccctgc	cgaacctacc
1801	cgccattctt	cctgaatgcc	tggcagtacc	tcagtgatga	cagtgcacgg	caggatttaa
1861	atgccgatac	acaccgttat	gacccgctcg	gccgggaata	ccaggtgaga	accgccaagg
1921	ggtatctgcg	ccaaaatcgg	ctgacccccct	ggtttgtggt	gaatgaggat	gaaaacgaca
1981	cgctctctta	attaacacga	taacgtttaa	taatcacacc	ttcctgccag	tcacggggga
2041	aggttaacta	ctctatcaag	gaaagggttt	atgactgtaa	acagaggcga	taacctgcat
2101	caaaaaacgc	cggaagtgc	ggttctggat	aaccgggggc	tgaccgttcg	cgagctccgt
2161	tatcaccgcc	acccaaatac	ccccaccacc	accgatgaac	ggatcacccg	ccatcgggtt
2221	actctctcag	gtcagttggc	gcacagcatt	gacccgcgtc	tgtttgactt	acagcagacg
2281	gataatcacg	tcaatcctaa	catgatttat	gatactgcac	tgaccggtga	ggttgtgcgc
2341	acaaggagtg	tcgatgcggg	taatgatctg	atattgaatg	acattaccgg	ccggcctgtg
2401	ctggccatca	atgcaaccga	agtcactcgt	acgtggcaat	atgagaatga	cactttaccc
2461	ggacgcccgc	tcagtatcac	agaacagcct	gctggcgaag	caggccgtat	cacagagcgt
2521	tttgtctggg	cagggaacag	tcaggcggag	aagaacagca	acctggccgg	acagtgcgtg
2581	cgctactatg	acaccgcccg	actgaaccag	acggacagta	ttgcgcttaa	cggcataccg
2641	ctgtccgtca	cgcgccagct	gctgcgggat	ggtacggacg	cagactggca	gggaaacaat
2701	gaaccgcgct	ggaacgaccg	gctggcaccg	gaaaacttca	ccaccctgag	cacggcggat
2761	gccaccggcg	cggtactgac	caccaccgat	gcggccggta	acctgcagcg	tgtggcgtat
2821	gacgtagcag	gcctgctgac	tggcagttgg	ctgcggcttg	cgggcgggac	agagcaggtt
2881	atcgtgaaat	ccctgacgta	ttccgcggcg	ggtcagaaac	tgcgcggaag	gcacggcaac
2941	ggcgtggtga	ccacctacac	ctacgagccg	gagaccagc	gccttggttg	cataaaaacc
3001	aaacgcccac	agggacatgc	acaggggacg	aaggtgttgc	aggacctgcg	ctatgagtac
3061	gacccggttg	ggaacgtggt	gaaagtgcag	aacgatgcgg	aggttacccg	cttctggcgc
3121	aacccaaaag	tggtgccgga	gaacacctat	gtctatgaca	gcctgtatca	gctggtcagt
3181	gccaccgggc	gcgaaatggc	caatatcggt	caacaaagca	cgctgttacc	cactccttcc
3241	ctcattgata	gcagtacctt	cagcaactat	tcccgcacct	acaattatga	ccgtggggac
3301	aatctgacgc	agatacgtca	cagtgtcccg	gccactggta	acagttacac	cacggacatc
3361	acgggtctcag	atcacagcaa	cggggcagtg	ttggacacgc	tgacggatga	tcgggcaaa
3421	gtggatgcac	ttttcactgc	ggcgggggcg	cagatccca	tgcaaccggg	caggaaacctc
3481	gtctggacgc	cgcgcggtga	gctgctgaaa	gtggcaccgc	tggtacgtga	cgggcagatt
3541	tccgaccagg	aatcctatcg	ttatgatgcc	gccagtcagc	gcatcatcaa	aaccacggtt
3601	cagcagacgg	ctaacagctc	gcaggcgcag	agcacgctgt	acctgccagg	gctggagcgg
3661	cacaccacaa	taaatggcac	gacggtgaaa	tgggtgctac	acgttatcac	gataggcgag
3721	gcgggcccgtg	cgcaggtgcg	ctatctgcac	tgggagaacg	gaaagccggg	tgccatcagt
3781	aacaaccaga	tgcgctacag	ctatgataac	cttatcggca	gcagcggtct	ggaggtggac
3841	ggtgacggac	aaattatcag	tatggaagaa	tactaccctg	acgggggcac	tgcggtgtgg
3901	acggcgagga	gtcagacaga	ggctgattac	aagactgtgc	gttactcagg	caaggagcgg

Fig. 2



3/14

chrim5ed2.seq

```

3961 gatgcaacgg ggctgtatta ttacggctac cgggtattacc agccgtgggc ggggagctgg
4021 ctgagtgcgg acccggcggg cactatcgac gggctgaacc tgtaccgcat ggtcaggaat
4081 aaccggcgga cactggatga taaaaacgga ctacgcgccg gaaatagata tgtatttttt
4141 ccattttatc atgaggacag gatttttcgt ctggcaagcg cgaatgttta cagaacggaa
4201 cataataaat ctgacatcat tgcggttgta gaagataaag cattagatag taaactatct
4261 accaatagta ttgagcagtt tttcaaaaaa cctaaaggaa aagcaatcct gaaaggatcc
4321 cctgatatta aagaaaggct actcaataat atagtacatg acctgagcaa tatgcaggta
4381 ggagatcagc tgtatgtaaa cgctcatggt cattctgcga aaccattttt ttactccgat
4441 tcgggatatt caaaaatcat caatggaacag ctccaaagag gggctaacta tgtagctaaa
4501 gatttagtaa ataagtttaa attaccagaa aatgcaacaa tcaagataag tacgtgtcat
4561 agtgctgaag gtaagggcgc tcatattacc gtcacatcca ctggaacaaa tgaaaaaatg
4621 agatacagtt ccattataga gaacaaaggg gaattttccc ggtcttttagc aggtaccatg
4681 gaaaatgagt taattaaact taattaaact agagttcgcg ggaatgtata tggttatctt
4741 ggcgcgacaa cgttctatgg tgctaaaaat gaaaaagtca tacacctcaa agatggcaat
4801 ctgacaactg cgttcatga aggcaagtta tcaatgttta ctaaaaagaa ccgattttca
4861 gaaaacattt ttgggttaaa ggtaaaaaga agtctgacgc gaacaaactt taccggcagc
4921 ggcgataaaa aacaaatttc aaaccgcatt taaatacggg cagccagcgc cagccagcgc
4981 cgacctgacc tgtcacgtcg ttgcttcccc gttacaccgg caggtcggac gctgctggtg
5041 cttttaccgg ttttactcgg atattgaccg gcataccatg agcatcacga ccaaatgtgg
5101 ttctgtcgca ttaacggcga gttaatcaga gatcccgcga gtggttggtt tttatactgc
5161 caatcgataa aattgttccg ccgcgcgataa acattcacct tcctcttgcg aatacgggtc
5221 tttccggagc atggcgacga gttctatccc tgccagtcac gtttggtccc ggcgaaacga
5281 tttaaaacct aacattgggtc gtatccgacg tttgacatgg cgatgggtctt gctcaatgcg
5341 gttattcagg tattttatctt gccggatttc aatcccctgc tgaggtctac cttccgcatt
5401 gaggagggtg agcygcggcg tatttgcccc acttttatcg atagtacca cagtccgtct
5461 gcccgtcggg atcaaccgac cggtaaagggt atttccactg gcctttgact ttaatgtatg
5521 tttcatccat tcgccatcgg gagccaacag gctttttcgc tttccggaaa gccttgctga
5581 gcaggggtac caaacgtaac acccagcgag gtatcgtggc gtggctcagc aagatcccc
5641 gttttgccat catttctctc agattacgta agctcagcgc gtaggtcaga gaccaacgga
5701 cacattgggc catgatatcg acaggataat ggaggagttt gaatgcgttt cggatcaggg
5761 acatcactct ggaactcactc aaaaacagga gagcttacct gatattacgc taatgcgaca
5821 gaacctatct tatcgattga ctgcataaat agtcacattt cccacctcog taaaaactt
5881 ctctgttaat gcgacagaac cgttttttcc atattgcctt gcatgctctg tagtgcctt
5941 aaattccttt aaatttctta tagttgctta aaatatccat aatattgcct gagccgattg
6001 gcgaagcgcg tcatattgagc cggtcggcga ggcgcgtcag caaatccatc cgctcgtgaa
6061 aaatcgccac aattaacgca ggcgcatttt caccgcggcag acaaaacaca tagtgactgt
6121 cacaatgcgc catgcgcaat gcgggaaaga ggcgcgtcat gtccttaaaa gagccttggc
6181 cgacagcaag ccgcgcaatg ccctgttcca gcgcggtgat gtagcggcgc acctgcgcgt
6241 caccaccattg ctgcgcgta tggcggatga ttgcgcgtaa atcggcctca gccgccttcg
6301 tgagtacata gtccatcagg cgcggtcgct cccggcaagt tcttcatcga gaatttcgcc
6361 gatacttttc ccgcaacctc tcccctccat cccttcattg atgcgtgtat ccagcaaggc
6421 tttcagctcc tgccatgcct gatcgctatc gctcattccg ggaacaggc gttcagagtgc
6481 gtattgcttg atcgtcttgc cttgcaaggc agccaatgcc ttcaggcttt ggtgctgccc
6541 gtcggtgatg tcaatggtca gacggctcat ggttttctcc ttatcggtta tacacaacc
6601 cacattagca catataatgg tttgtggcta tttattacac aggggggtcag atatcgtttt
6661 gtccggcaaaa cattcgtttg tcggtcattg tcattttcag aagacgactg caccaattaa
6721 tagggattag agcctggtgt gcatatgact ggcactgttg caaagtttga gtcactgatt
6781 atgctaagtt aaatgtttcg taacgggagc tcaccgtatg aatgtattca aaggccgtga
6841 ttttacaggc attatcattt tgtgggcagt tcggttggtac tgcaaatacg gcatcagcta
6901 tcgtgaactc caggaatgct tcaactgagc tggcgtaaat gttgaccata cgactcttta
6961 tggttctggt gcaataagta aagcctcggg aatatctcac ttatttgttg ataagagtgt
7021 cgtaaatgtc gttgatacga aaagccttca tgacacccat gcataagaga agcccatcat
7081 taccaggggc ggtattcctg ccgcctgagg gtttctgctg attatccctt tctgcctttt
7141 cccgcattct gttagaatgc ccacttctta attgtttcca aaactaatgt tgttatgtca
7201 aatcaagatc ctcataataa acgggacagt ctgttctctg cccaatcgc caatttaggc
7261 gactggagtt tcgacgaacg tgttgccgaa gtctttcctg atatggtgaa acgttccata
7321 cccggttatt ccaatatcat ctccatgata ggtatgctgg ccagtcgctt cgtgacgcca
7381 ggtagccaaa tttatgatct cggttgctcc cttggggcgg caactctgtc catccgcccg
7441 agtatcaatg ctgataattg ccggtattatc gctatcgata attcaccagc catgatcgaa
7501 gcgtgcgcgc gccatattga ttcttttaag gccagtacac ccgttgaggt gatcgaaag
7561 aatatccttg ataccgacat tcaaaatgcc tcgatggtag ttttgaaatt cactattaca
7621 ttcttgaccc ctgatgatcg ccagaaaata ttgaagaaaa tttacgcagg attaaaacc
7681 ggaggggttt tggttctgtc tgaaaaattc aattttgaag accagaaatg tggcgagtta
7741 ctattcaata tgcaccatga tttcaagcga atagtgcgtt atagtgcgtc ggaagtgcag
7801 caaaagcgca gtatgctgga aaatgtcatg cggacagatt ctgttgacac ccatagtcga
7861 cgccttaaa aagtcggttt ccagcatgta gaagtctggt tccagtgttt caatttcggt
7921 tcattattgg caataaaaag aactgaacaa tgatcgattt cggtaatatt tatcaattga
7981 tcgccaagca cccactaaac cattggtctg atagcctgcc agcacaattg agccactggc
8041 aaaaaacatc acagcacggt cagttcagct atgggtgcat aatttcggaa aatttcgctg
8101 agatcaagcc aagccacctt gacctgaaaa atgggtgcat tgcgattcat gagccggatc
8161 tgtcaaaagg tgaaaaagct cgcctccaca atatcctgaa ccatggcgaa ccatggcgaa
8221 aaggcccttt ttcattgtat gacgttgaaa ttgataccga atggcgctct gactggaaat

```

Fig. 2(i)

SUBSTITUTE SHEET (RULE 26)

4/14

chr5ed2.seq

8281	gggagcgagt	gctgccccat	atctctcctt	tagaaggaaa	aaccgtactt	gatgtcggct
8341	gtggcagtg	ttatcacatg	tggcgcatgg	ttggcgaagg	cgctcaattg	gttggtgggta
8401	tcgatccaac	ccaacttttt	ctctgtcaat	ttgaagcgat	cagaaagtgt	ttggggaaca
8461	atcaacgagc	ccaccttctg	ccattgggca	tcgaacaatt	acccgaactg	caagcctttg
8521	atagcgtatt	ttcaatggga	gtgctctacc	accgcccgtc	acctcttgat	catctgtggc
8581	aactgaaaaa	tcaactgggtg	tctgatgggtg	agttagtgtc	ggaaagttaa	gtgattgagg
8641	gtgatgaaaa	tcagtgcctc	attccgggtg	aacgctatgc	acaaatgcgg	aatgtctact
8701	ttattccctc	ggccaagatg	ctgaaagtct	ggctggaaaa	atgtggtttt	gtcgtatgtca
8761	gaattgtcga	tcattgcggct	acaacacctg	atgaacagcg	ccggacagaa	tggatgaaga
8821	ccgaatcact	ggtagatttc	cttgacctat	cagatcacag	taaaacaatt	gaaggctacc
8881	ctgccccatt	gcgtgctgtc	ctcattggcc	gcaaaccata	atattgaata	aatattaatg
8941	agtgaactgt	ccaatatggc	aattcactca	ttaagtctta	agatttcgct	ttccttatga
9001	cgcaagcgat	atcacatcta	ccgttaaatc	aggctcatca	ctcccttcac	cgactcaact
9061	aactcaccat	caacactgta	gtgagaatat	tcattctacat	cacgatcgct	cgaactcatc
9121	gccaccctta	cctttcggtg	cttcattggtg	gaaggtaaca	ttttaccgct	cgagctatga
9181	acttccgtca	ttccggcttc	cagaaactta	ctgatattac	tcaccctgac	acccgcgccc
9241	ggcataatta	tcggccccag	gctggcttgc	atcagctctt	ttaacagcgt	cagccccagt
9301	tcgacattct	gctgttggtc	tgatgtttaa	atagcttgca	ctccaagctc	tgctcagttgt
9361	tccaacgcaa	catgcggatt	aaaacacata	tcaaaagcgc	gatgaaaagt	aacagccata
9421	tttcccgaca	gtgacatcaa	ctgccgcata	cggagtctgt	caatatggcc	gttttcgctc
9481	aaaatgccaa	aaacaatgcc	ggggaaacccc	atatcacgga	tacgagcaac	gtcatttttc
9541	atggcttcaa	aatccatggt	gtttaaacag	aagtctcccc	ctcttggccc	cacaatggga
9601	tgcacaggaa	tagataaccg	ctgtaacgac	tggtgtaatg	ccccaaaact	gggtgtcaat
9661	ccgccttcca	acgggcttgc	gcttaattcg	attcggctcag	cgccccgatt	ttgtgcaacc
9721	agcgcacagc	ttatgtctata	acagcaaaat	tccagcttta	tcattaaaaa	gccctccgaa
9781	tacgaattcg	tcaacaagtc	atcatttaac	ctaaaactac	tttattagt	aacttattaa
9841	ctatgacaac	taactttatc	taatgacatg	ttggaaatca	caaggtcaga	attttttact
9901	ggaacagcat	gataaaaaac	tcatttttgc	cggctatacc	tcacttttga	caattttctc
9961	gatacaaaaa	ggatgatatt	tgatcgtgat	ttcaccatca	gtgacagcca	aaatcggggt
10021	cggcaaccgt	tcatttttttc	cttttggtatg	actctccttc	atccgaaaaa	tatctggtaa
10081	aggttgaggc	aataactttt	tagcttcaca	aataagttgc	tctggtggtg	caggcagcac
10141	cagttcgaca	tggtcccaac	cttggtggac	atagtgtttt	tggtggggtg	aaggcaattc
10201	cagcaaatca	attttccagt	caagtagtac	tattggctgg	ttcagatcaa	ataagcagat
10261	cggacggcca	ttaatgatat	tttcagatat	taactgacca	cattgcagaa	agcccttacg
10321	ccaacgatcg	gctattttac	tttcattaca	acgcagggaa	atatggtctg	ctgaatatgt
10381	ttcgaagtgt	aagccaagat	gcccttcaaa	ttgcttaagt	ttttgtctaa	atccggctaa
10441	atcagccact	aaatcctgta	actcagaat	ttttgaaaaa	tgagacattt	cttccccatt
10501	aaataaaacc	taaaaattgc	gttccattaa	ttgtacataa	atcacaaatc	gtctattttt
10561	gccgaaatat	cactcagtaa	gcgactaatt	gaagtgtggc	taacgcagaa	tcgectgaaa
10621	gacaggctaa	aaacaaaaag	taacaccacc	agaggtggct	gatggtagca	tgacggaccc
10681	cgaatatggt	ataaaccccc	tatttttctc	ataaccaca	cgcttaaaag	tattgcattt
10741	ccaaaatgca	taagctttcg	tgcgtaactt	aaggtaaacac	ggtgaaatata	caggttattc
10801	tttcagaaaa	aatcagcaat	gcgctgattg	aagctggcgc	tccaaccgac	agtgaagctc
10861	acgtccgtca	atctgccaac	gcacaatttg	gtgactatca	agcgaatggt	gtgatggctg
10921	ccgctaataa	ggtgggaata	cctcctcgac	aattggcaga	aaaagtcgtc	agccaactgg
10981	atctgcaagg	aattggcagc	aaagtggaaa	ttgcaggccc	aggtttttatc	aatatttttc
11041	ttgataaagc	gtgggttgca	gcaaatatag	aaactaccct	gaaagatgaa	aagctcggta
11101	tcaccccgat	ggaaccgcaa	accatcgtaa	tcgattattc	cgcacccgaat	gtcgccaagc
11161	agatgcattg	tggaacacctg	cgctcaacca	tcattggcga	tgctgcggcg	cgtacccttg
11221	agtttcttgg	gcataaaagt	attcagacca	accacgttgg	tgattgggga	accagttctg
11281	ggatgctgat	cgcttatctg	gaaaagatcc	agaacgaaaa	tgccaatgac	atggcattag
11341	cggatttaga	agctttctat	cgcgaaagca	agaaacacta	cgatgaagat	gaagagtttg
11401	ctattcgcgc	tcgttaactac	gtcgtcaaac	tgcaaggcgg	tgatgaatat	tgccgttaaga
11461	tgtggcgtaa	gctggtagat	atcacctatg	ccggaatca	ggaaacttat	aaccgcttga
11521	atgtcacatt	gacagaaaaa	gacgttatgg	gtgaaagcct	gtataacgat	atgctaccgg
11581	gtatcgttgc	agatttaaaa	caacgtggaa	ttgccgttaa	gagtgatggc	gcgacagttg
11641	tttaccttga	tgaattcaag	aataaagaag	gcgaaccat	gggcgttatt	atccagaaaa
11701	aagatggtgg	ctatctttac	accacgacgg	atatcgccctg	cgccaaatac	cgcatgaaa
11761	ccctaaatgc	cagccgtgtg	ctttactaca	tcgattcacg	ccagcaccag	cacctgatgc
11821	aagcttgggc	aattgtacgg	aaaacgggtt	atattccaga	atccatgtca	ctcgaacacc
11881	acatgtttgg	catgatgctg	ggcaaatgag	gtaaacattt	caaaacccgt	gccggcggca
11941	cagtaagact	gtccgatttg	ctggatgaag	cgattgagcg	tgccgatacc	ctcattctgt
12001	agaaaaaccc	agatatgcca	gaagacgaac	tgaaaaaagt	cgtgggaagc	gtagggattg
12061	gcgcgggtga	atatgcagat	ctttccaaga	gccgtactac	agactatgtt	ttcgactggg
12121	ataatatgct	ggcctttgaa	ggcaaacacg	caccttatat	gcaataacgc	tacacgcggc
12181	tgatcatctat	ctttaaactg	cgggatattc	atgaaaaacg	cctgacactg	ccggtgatgc
12241	tgaatgaaga	acgcgagcag	gcattggcaa	cccgcctgtt	gcagtttgaa	gaaacgatca
12301	ctaccgtcgc	ccgtgaaggt	acgccacatg	ttatgtgtgc	atacctgtac	gatctggccg
12361	gtctgttctc	tggtttctat	gagcactgcc	ctatcctgaa	tgccgatagc	gaagaactgc
12421	ccgagagccg	cctgaagtgt	gctctgtcga	cagcgaaaac	tttgaagcaa	ggtccttgata
12481	ctctgggtat	tcagactgta	gaacgtatgt	aataatcttc	tacaagctg	aaaactggcg
12541	tgatattatt	ttattacgcc	agttttttcc	tttcttctat	tctgtcaaaa	attaccaatc

Fig. 2(ii)

5/14

chrim5ed2.seq

12601	tgacatatata	ttaatcttga	gctaacattt	tgatatTTTaa	tcatagaaat	atatgaacac
12661	agaagcagtt	gttatgaaaa	aattattttat	ttctaactgc	tagccctacc	aatctccata
12721	aaaaaacctg	attttttattc	acttaatgata	attaatgata	atttctattt	taattaacct
12781	tgttataaaa	aatagtattt	taaaaaaaca	ttttacatta	tataaaatat	atcaatcgac
12841	tctttatttc	tttatccatt	tataaaatat	attttttacc	aaaataatat	ttaaatacata
12901	tatttatattt	acatcacggt	agatcaaaat	aacaattttt	tagtcggtta	cccagattca
12961	gaacggcacg	atgatatata	agtcacatgg	gttgtaaaata	aaggaaaaag	ataaaaaaga
13021	ggagataaat	cttcgcatTT	cttcaatgaa	gatggatata	gatactgtaa	gtagtaatt
13081	taaaatgaaat	ccattaacaa	attaatattt	aattttacatt	aagagaggat	tctatgagtg
13141	attggactgg	tgtttcaaca	tttaatgtta	ttcttgaaac	aggattagat	aactgcaata
13201	tctacgctaa	tgggcttaac	atgattgggg	taattattaa	tatcacaccc	actgatgatg
13261	aagggaactt	cgtagatatt	gcagatgtta	cactaaatga	taactacaag	attgttgatt
13321	atatcgatgg	aagcgacatt	gatggcagtg	acggatgggt	ttatacagga	aatcctaattg
13381	aatacaacac	tattcctaaat	agtcagtcct	attcctttatt	aaagagtgaa	aattctcaaaa
13441	ttacgcaaat	taaacgatat	gtttcctgtt	caaatacatc	caggctaaga	accaagtctt
13501	tttctgcgaa	ggttaaccact	accagtggaa	aagttatttc	aatagctcaa	ggtagtaatt
13561	attcatctcg	ggtagtaatt	aatgcaatag	atgcaactaa	ttttactgat	gatgaacttc
13621	gaacaacaaa	agaaaacagg	tttgaaaatc	aatcctatac	gtcacataaa	tcatctacaa
13681	actctttata	tgtgcatacg	tggacaatac	caagaagctt	aaaactacaa	aattggcggt
13741	gggaagatta	caataatggc	tggacttggg	cacaaaagtg	ctactataaa	acaggagccg
13801	atggaggatc	agagtcaacc	cgctgggttg	ccgctgggtc	aatctttcca	ccaggaaatt
13861	atgatggcct	gtggctagat	aatgatattc	cactaagtgg	tatggcacac	aaaagctaca
13921	atggtgatac	tggatcaaat	caattgagtt	ttaccgctat	tataggttaa	ggtttcagct
13981	gggtttataa	tatatccgga	cttgatagag	ggcatgccgt	tattattatc	gaccagtagt
14041	gtaacaaata	tagaatatta	ttccatgccg	ggtatgaaaa	ctcagatcc	tactgtctt
14101	catcaatagt	atattaaaaa	tatgtgtacc	aatgggtgtg	tagcatTTcc	ccaatacgaa
14161	acttgacctt	gtggctgtta	ttataattta	ttttcacagc	cactttttaa	gtatacataa
14221	aattccttaa	gttattcagg	agaataacca	tgagtgacaa	taattgagtt	tttactcaag
14281	ctaataattt	caccagcgct	gtcagtggtg	gcgttgaccc	tcgcacagga	ttatacaata
14341	tacaaattac	tttaggtcac	attgttggtg	acggtaatct	tggaccctact	ctgcctctta
14401	ccttaagcta	ttctcctctt	aacaaaacag	atattggatt	tggcgttggt	tttaattttg
14461	gattatcagt	ctacgacaga	aaaaattctt	tattgtctct	ttctaccggt	gaaaattata
14521	aagtcatcga	aaccgataaa	acagtaaaac	ttcagcaaaa	aaaactcgac	aatttacgct
14581	ttgaaaaaga	cctaaaagaa	aattgtttatc	gtattataca	taaatccggt	gatattgaag
14641	tgttaactgg	tttcaataac	aatgcctttg	acctgaaaag	ccctaaaaaa	ctattaaacc
14701	ctgctggaca	tgctatctat	attgattgga	attttgaggc	aactcaacct	aggctaaatc
14761	gtattttatga	tgatctggat	gggcatgata	taccattatt	aaacctagaa	tatcaaggac
14821	taattaaaaac	gatattaacg	cttttccctg	ggcaaaaagga	aggctaccgt	accgagctac
14881	gctttctaaa	cagacaattg	aacagcatcc	acaactttag	cttgggttaat	gaaaaccctc
14941	tcacttggtc	cttcggttat	acccctatag	gaaaaaatgg	tattttgggg	caatggataa
15001	caagtatgac	cgctcctgga	ggattaanaag	aaacggttaa	ttatagtaat	aataatcagg
15061	ggcatcattt	cccccaatca	gccaatctac	cggtgttgcc	ctatgtcaca	ttaatgaagc
15121	aggttcctgg	agcaggacaa	cccgctatac	aagcagaata	ttcgtatacc	ttcataaatt
15181	atgtcgggtg	gggatctaatt	ggatatatgga	ataataaatt	agataaatctg	tatggattga
15241	tgacagaata	taattatggc	tctactgaat	cccgcagata	taaagataaa	gaaggccatg
15301	atcaaatagt	ccgtatagaa	cgcacataca	ataattacca	tctgttaact	tccgaattga
15361	agcaacaaaa	tggatatata	cagacaaatg	agacagcata	ttatgtctatt	tttgccata
15421	attttgattc	tcagccctca	caattccagt	tgccaaaaac	caaaacagaa	acttggcgta
15481	gtgcagataa	cagctatcga	agtgaattta	ctgaaaccac	atttgatgaa	agcggaaacc
15541	ccctaaccac	agtaatcaaa	gataagaaaa	cacaaaaaat	aatctcccc	tcaagcatt
15601	gggaatacta	ccctcggct	ggggaggtcg	ataattgccc	accagaaccg	tatggattta
15661	ctcgtttcgt	aaaaaaaatc	atacaaaact	cctatgactc	cgaatttaaa	gatgatccgg
15721	agaaatttat	ccagtatcgt	tatagcctca	ttggcagtc	gagtcattgt	actttaaaaa
15781	tagaagagcg	ccactacagt	gcaactcaac	ttctgaatag	tactctattt	caatataata
15841	cggataaaaag	tgaacttggt	cgtttatttaa	aacaaaactga	atgtaccaaa	ggagaaaatg
15901	gaaaaactta	ttctgtcgtg	cataaattta	cctatacaaa	acaggacgac	acgctgcaac
15961	agagccattc	cataaccacc	catgataaatt	tcacaattca	ccgcagtcag	gttcgttccc
16021	gttataccgg	gcgtctgttt	tctgacacag	atactaaaga	cattgtaact	caaatgtccc
16081	atgacaaaatt	gggtcgatta	ctcacacgca	cccttaattc	cggtacacca	tatgccaaca
16141	ctctgacata	tgattatgaa	ctaaataatc	ttcaggatga	caatcgccct	ccgtttgtta
16201	ttaccaccac	ggtatgaaat	ggcaatcagc	ttcgcaatga	attcgacggt	gccggacggc
16261	atgtcagcca	atgcctgaaa	gactccgatg	gtgatggaaa	attctatagc	atacatacgc
16321	aaCaatatga	tgaacaaggg	gctcatcata	catctacata	ctccgactac	ctcacaaaatg
16381	gaagacaaca	gacggatcct	gataaggtgc	atctgtctat	gtcaaaatcc	tatgataatt
16441	gggggcaaat	tgcgaacaca	cactggagtt	atgggggttc	agaaaaata	actgtagatc
16501	cgataacatt	gacggccacc	aaacagttac	aaagcaatag	caataatgtg	caaacgggta
16561	aagaggttac	aacttatacg	ccaagtcaac	aactatatac	gattacgtta	tttgacgaag
16621	caggccattt	acagagttgt	cataccctga	ctcgggatgg	ctgggatagg	gttcgcaaaag
16681	aaaccgatgc	aataggccaa	tgcactattt	accaatatga	taactataac	cgagtcattc
16741	aaataacgct	tcttgatggc	accatcgtaa	atcgcaaaata	tgcacccttt	agtagtgata
16801	cgctgataac	agatatcgcg	gtgaatggaa	tttccttggg	acagcaaacg	tttgacgggt
16861	tgagtcgatt	aacacaaaag	caagatgggg	gacgagtatg	ggcttatact	tattcggcag

Fig. 2(iii)

6/14

chrim5ed2.seq

```

16921 gtaatgacca atgccccatca acagtaataaa caccagatgg tcagttttatc cattatcaat
16981 atcagccaga attagatgat gcagtagtatc aagtagcatc aaatgaaatt actcagcagt
17041 tcagctataaa cccagtcact ggggcattat taaaggcggg ggcagaggga caaagcttga
17101 cccctatcta ttatccatcg ggaagactta agatggaaaa tatcaatgat atgaaaaaaa
17161 tgagttacct atggacactt aggggtctgg agaacggtta cactgatctg actggaacaa
17221 tacagaaaat ttcgcgtgat acccatggca ggggtgacaca aattaaagat tcgtcaataa
17281 agactactct aaattacgat gacctgaatc gccatattgg tagtcaagta acagatttag
17341 cgactggtca tatgttgaca acaacagtg gcaatttgatg cttaaaccga gaaattggac
17401 ggaaaattgtg tgatagctca ggccatagct tagatatcca gcagagctgg ctgaaaacac
17461 agcaatttagc aaatagaata gtgaaactga atggagtatt gcagcgtaca gaacagtact
17521 cttacgattc ccgtaatagg gtgaaccaat ataaatgtga cgggtgcgga tgcccgcagc
17581 acaaatatgg ccatagcata gtcacacaaa attttactta tgatatctat ggcaatatca
17641 ccgcctgtca caccacattc gcagatggga cagaagacca tgctaccttc aaatttgcca
17701 acccaactga cccatgccaa ccagcagagg tacaccacac tcatcagata atgccggata
17761 atatcaggct gaaatatgat aaggctggta gagtaataaa tatcactgat aacctaggaa
17821 atacggaaaa ctttacctac gatacattgg gcagattaca aaacggtcaa ggtagtggtt
17881 atggttatga tccattaaat cgcttagtga gtcagaaaaa agatacccta gattgtgagc
17941 tgtactatcg ggaaaccaat ttgggtcaat aagtagcga tgggaaatg atccgtttat
18001 tacggacggg tgaacaata atcgcacagc aacgcgcac aaaagtcttg ctaacaggaa
18061 cagatagcca acagagcgtg atattaacga gtgataaaca aaacttgtct caagaagcat
18121 atagtgcata tggaaagcat aaatctacag caaatgacgc ttctatcctg ggctataatg
18181 gtgaacggc tgacccagtt agtggagtga cacatttagg taatggttac cgctcctatg
18241 atccaacatt aatgcgcttc catactccag atagcttaag ccccttttgt gctggaggga
18301 ttaatcccta ttctctattg ttaggagacc caattaatcg ctacagacct tctggtcatt
18361 tgagttggca agcatggaca gggattggca tggggatcgc tggattactg ctgaccatag
18421 cgacaggtgg aatggcaatt gcagcagcgg gaggtattgc ggcgcgaatt gcttccacct
18481 ccacaactgc actggcattt ggggactgta gtgttacatc ggatataacg tctattgtta
18541 gcggtgcact ggaagatgct tcaccgaagg catcttctat actcggatgg gtttcaatgg
18601 gaatgggtgc tgccgggtta gctgaatcgg ccattaaagg tggcaccaaa cttgcgacac
18661 atctaggagc attcgctgag gacggggaaa acgccttact taaatcgact tccgaaagtt
18721 ctagaataaa gtggggagtg acaagaagct tagatagaga aattgttcgc aatgaagaag
18781 gtcagggtgat aaaagatcat agccgaggtt ataccgataa ctttatgggg aaaggagagc
18841 aggtatatt agttcatatg gataaagatg gatttttcta tcatacagaa ggaacacaa
18901 ataattggaa agggccatac actcgacata ctctgaaaca atcgttgtat tatttgaaag
18961 acaataacat cgttgatctt acacaaggag gagacaaacc tgttcattta ttatcctgct
19021 atggaaaaag cagcgggtga gcagataaaa tggcaaaaaa tatcaacagg ccagttatcg
19081 cttattctaa taaaccaaac atatacaca gattagccag aatagaaaga aaggactttt
19141 tcttaaaaaa tacttaccat tcgtatgatc cagggaagat catactggga agaacagaaa
19201 aaacagtgaa accaaaaact ttctgcccct aataaccttg caaaattcaa aggtactggc
19261 agaagtcata taaataactc tatgactggg taaatgaaat cagttattcaa acattaaaaa
19321 ggatggaaat ggtcatctta tcaaccgccc aataaaaaat tgggcggttg taatgaataa
19381 aattatttat taatcaggga atcactgcaa tccccgatga gcaaatcctt tcagtctaaa
19441 tcccatcaag gccagaactg caaaaataact gcctgctccc gcaatcacta cgcccattaa
19501 gcgcagtagg cgcattgcca tattgcccgt ttcccattgct ggcataaccc acaatactgc
19561 caacagcacc ccgaccatca cagcaattgc caccaccaat ttaaccacca atatcccca
19621 tcccgcacaa ggctgtaaaa tatcacgctt acgcagttgc caataaagca tactggcatt
19681 gaagcaggca gccagaccaa tagaaagcgc cagacctgca tgtttcaacg ggccaacgaa
19741 agcaagggtc atcaattggg tcagaatcaa ggtcgcgatc gcaattttta ctggtgtttt
19801 gatattctga cgtgaataaa agcccggagc gagaacttta accacaatca acccttcaa
19861 gccaaaacag taggcaatta acgcccgtg agtcatctca gcatcaaaa gcaaaaagt
19921 accatattga aataatgata ccgtcagagg ctccgcgaga ataccgagag caactgcaca
19981 aggcaacgcc agcaagaaac agagacgtag ccccaatcc atcagttttc gatattcttc
20041 gtgattacca ctggaaaaac ttttcgccc gtaaggcagc aaaatcgctc ctaacgccac
20101 acccagtaca ccagaaggca attccattaa acgatcagcg taatacatcc atgaaacaga
20161 gcctgaaacc agaaatgagg caaaaattgt attaatgatc aaggaaatct gcccgaccga
20221 tacacccaga attgcaggcc ccatttgacg gataaccgcg catacggcac tgtcacggaa
20281 agaaatccgc ggcaatacca gcatgtcgat ctttttcaga tgaggaaagt gataggccaa
20341 ttgcaaaaac cctccggcaa caacggccca acccagcgcc agcactggcg gattgcaata
20401 aggagccaca aacaatgcaa aaatgatcat actgacattg agcagtgtcg gagcaaaagc
20461 aggcaccgaa aagcgggttc atgtattaa gattgcgcca gccaaagaag ccagcgaat
20521 caaaaagata taaggaaacg taattctaag taaatcacgg gtttaagcaa atgtatccgg
20581 tgtatccgta aatcccggcg cagtcacata gatgatccaa ggtgcagcaa ttacacctat
20641 cactgagacg atagccagaa tcaatgtcaa catacctgag atatattgcaa taaaggtacg
20701 tgttgcttca tcccctgtt gatttttcta ttcggcaaga ataggaacaa aagcttgcca
20761 aaaaacgccc tctgcaaaag tacggcgtaa caggttaggt aatttaaagg caacaaaaaa
20821 ggcatccgtc gccattcctg caccaaatat atggcatcac agtgaattca gaataaagcc
20881 cagcacgcga gaaaacatcg tcattgaact gaccgctgcc agtgatttca ataagttcat
20941 ggtattgttc taaagttgta ttcttatgga attaagcata gctatcccat gctatcccat
21001 caggcatcat aaaaatggca tataaagcaa ttggcgagg tagcagccgg tgtttaaagt
21061 ctaacagaca aaaacccgtg ctacattttc tatattacgc cccattcagg tttcccggag
21121 attcataaac ccactttgcc ccatgcacgt tcagtcaatg cctgcagcac atgatcttgc
21181 caaacaccat ctatcattaa ataattcttg gcataacctt cctgctcaaa tcccaacttt

```

Fig. 2(iv)

SUBSTITUTE SHEET (RULE 26)

7/14

```

chr15ed2.seq
21241 ttgagcaggt tccactacg atgggtatgt ggcatgtaat tagccataat ccggtgcatt
21301 ctctgatagc gctgcatata gcgatcgca ggttgcagcg cttcatacat caatccttgc
21361 ccttgcaatt tctcagctaa agaataacca agataacaag agtggaacgc gccgcgtaca
21421 acattagtaa aattcgccac acccataatt tcacgctcat cagagtccaa taatacaaaa
21481 ttaaatgtcg cattctgccc ttgtaactca cgcataatag tcaaccgatt tgcctatcca
21541 gagggttgat aaaaactgcc gtcccttggt ggctcccatg gcttcaggaa atctttatatt
21601 tctgaataat actcagccaa tcgataagca tcacgttcat ataccagacg aacaaccatc
21661 ctatccgtaa taaatcgaat tttgggcgat gcagaacgat aacaaacat gccttctcct
21721 gatatttgtc ttgaaaaaat tctggataat ttatctctta ttaaaattat tgctatttac
21781 cctacgataa aaaaatatca tctataaccc tctaccttaa agatgagatt agggtcagaa
21841 taataagaac ttcatattta attctctcat atttttagtg tgcgcaatgt cggtgggttc
21901 acaagcacgt agcttgggta aatacttccat gctatttgat aatttattag ttgttttagg
21961 ttttttgtc gtttttcccc taatttcaat tctgttcgtc gaacaacttg gctgggaggc
22021 attgattggt gggttcgctc tggggcttcg tcagcttggt cagcaaggct tggggatctt
22081 cgggtggcgc atcgagacc gatttgggtg caagccgatg attgttactg gcatgttatt
22141 gcgagcactg gggttgcctc tgatggcaat ggcacatgag ccatggatat cctgtcttcc
22201 ctgcttcta tcaggattgg gaggaacatt gttgatccc cccagagcgg ctttggctcat
22261 taagttaacc cgtcccatg agcaggggag ttttattca atcctgatga tgcaggacag
22321 cgcaggtgcc gtggttggcg cactcatcgg aagctgggtg ctgcaatatg atttcaatat
22381 cgtctgctgg attggtgcat ccattttctg gctggccgca ttattttagg ctgttctact
22441 gctgcatcac cgtatttcaa caatccgtac tccatcaaaa gaaggcatga tgcgggttat
22501 tagagatcgt cgttctcttt actatgtgct gacattgacg ggttattttg tctgtcagt
22561 acaagtgatg ctgatgtttc cgattattat ccatgaaatt accggcactc ctactgcgt
22621 caaatggatg tatgccattg aaacogctat ctccctgaca ttgctctatc ctactgcacg
22681 ctggagtgaa aaacatttcc gactggagca gcgcttgatg gcgggcttat tttgatgag
22741 catctgcatg tttccgatcg gctgggtcaa tcaattacat acactgtttg gcctgctttg
22801 cctgttttat ttaggtttgg taacagcga tctgtcgtg gaaacgtgga gtgcttact
22861 gctgatccca cggcgcgctg gcagttatat gggatttagc cgtctgggtt tagctctagg
22921 tgggtgcgata gggtacaccg gtggagggtg gctctatgat actggccgtg acttgaatat
22981 gccgcaatta ccttgatttt gtctaggatt gtctgggttg attaccattt atgctcttca
23041 tcgccaattc aatcagaaga aaattagctc tgtgatgctt ggtagacatt aatcctatta
23101 gaaatagtgt aaaatctgcc cattgaaaat aaaaaggagc caatatgaaa agatttttct
23161 taggggcagc attagtgtcg gtaggattag tcagcggctg tgatcaattt aaagacttca
23221 gcatacaaga aggtctgatg aatgattatt tgctcaaaaa agtgcattat cagaaaaaaa
23281 tcagcatccc aggtatcgcg aatgccaaata tcacgctggg ggatttatcc agccagatag
23341 gccgccagga tctgaaaaaa attgaactat ccacgcaagc aaaagttcaa cttgcaacac
23401 tactgggtac gattcaggct gatatagaac tcactatcaa ggctaaaccc gttatttgatg
23461 cagaaaaagg cgctatttct gtgaaaggcg tggaatcgt agactaccag acaaccacgg
23521 aaaaagcagc ggctccggtt aaggcattga ttccttatct gaatacctct ttgagttagt
23581 ttttcgatac tcatccggtt tatgttctga atccagaaaa aagcaaggcc gaggcagcag
23641 cctcacaatt cgctaaaagg ttgaaaaata aaccgggaa gttagtattt ggggtgaccg
23701 ataaataatt tttatcgtca ttgaaataat aagggcaaat tattaattga tttgttccc
23761 ttttattttt tcaattagtg accgatacac tcgcctgttc atgcagcaa atggccttcg gctgaactat
23821 tatcgtgaca ctgtcactca gattcctaatt catcacgcaa tgcttcaact gcttcacgag
23881 tgacaatcgc cagcgttcga taaaaaccac tgggtgcagt ggcttcaact ttaccgagga
23941 actgccaca ccaaggcaaa agatactcat caaacaacgt aatttgtgct tggatttcat
24001 cttcagctgc ctgatcttcc agccatgaca cagccagcaa tagagagccg aactgatcag
24061 caggagtatc agacagtggtc attccccgtt ctgtcaaaaa ctgacgtact tcatcttctt
24121 tctcgtccgt ataattcagt cgaacacagc caacactcgc cgattctcca gtaaacagag
24181 cgtgataatc ggctgccatc actgaacaat cactgttttg ctgtaaacga tccagccatt
24241 catcctgttc caaaggccat gctgttttta gtttcccctc agcaatcatg gtgattaaag
24301 gttgcaaaac gggatcttgc ggtgcgcggt taaacaatgt gcccaatatg cggcagacaa
24361 tagaaaattc gttcataaact tgattcaact ccaataaatt atcaaatagc gttattgtcc
24421 ctaatcgctt gccatgctgt aaagcaactt gttaatagcc acgtacccta tgcagaggc
24481 cagttggcat ttcaccctgt accatccgcc ggatattttc acagatgaca tccatcgag
24541 cctcaggaat agtggttagc gcaatatggg gagtcacatt aatacagggg tgagtccaga
24601 atgggtgcat atttgacaat ggttcttcgg caaacacatc aagggttgct cctgcgat
24661 aacctttatc aatagcaacg agcagatcct gttcgacaag ctgcgctcct cgcgccagat
24721 taatcacata tgaacccgat tttagtgtgc tgaataatga cagattaaag ataccacgag
24781 tgtcgggagt atcagggaga aggttaatca gacacttgca gccagaaaga aatcaccca
24841 attgctcttt accgtaaaaa ctttcgacac tatcgagttg ttttgagctc ctgtccaac
24901 aacgcacatt aaaatcaaat tccatttaatt tcccaatcac acttcgccct aatattccg
24961 cccctaaaac gccgataaca aattcttttc ggttatgcgg agcaataggc ttccaaaggc
25021 gctgttcttg gtatcgttta tattcatcca tgcggcgga ataatgcagc actgaggtaa
25081 tggcatattc ctgccatttc ctgtatcttc tgcgtatctc gaggcgatc aatggaacat
25141 cagccagtaa cgtaccggga ttttctgatt cttgtttgaa aatcgcatcg acaccggcac
25201 ccagtgcgaa tatgcctttt agccctgtac gattggctaa catttcataa ggtgggtgcc
25261 aaactaatgc atagtctgcy ggctcttgat caccactgac ccattgctc cctattggcat
25321 caggcagacg tgcctgaatt ccttgaatcc actcatcggt attaaaggaa ggggtgaaaa
25381 aaataatgtt catacaatct tattccttat tgtttttttg atagggttaa cctattgcta
25441 aatggttgc aagcctctgc tggaaagcga ggaatgaaaa tattaataat gttaccagat
25501 taacatatatt accactgcac attacaaaaa gcgcatcggc ttttagtaaat gactatcgaa

```

Fig. 2(v)

8/14

chrim5ed2.seq

```

25561 tattcaaat gtttttttatt tgtgtaatca gtcaaaaagc ctgaaaaaat cgtcataagc
25621 ctgttgacgc ctgccctgct tttccctata gtagcgcgcc gttgcagcga cgaactcaag
25681 tgatattcgt acaacaacaa aatcagggtga ggtgtccgag aggtcgaagg agcacgcctg
25741 gaaagtgtgt atacgtgaaa acgtatcgag ggttcgaacc cctctctcac cgccatattc
25801 taagaaagag cctgaacaca atactaaggc ttttttgagg ttactcttga tagagcattg
25861 aatctataat ttagtaaccc ttgcggaaaa tccctgacag gacgaccgca gaaacaaaac
25921 acggtgaggt gtccgagagg ctgaaggagc acgcttgaaa agtgtgtata cgtgaaaacg
25981 tatcgagggt tcgaaccctt ctctcaccgc catctttcaa gagaaagcct gaacttatgt
26041 tcaggctttt tgcattttat actcccaaaa gtatagggtga aaaacctcgc aagggttcac
26101 ctcaacaacc tgctctaatt ggaatgtctt aaagatttgt ggcttaatta caccttggtc
26161 tagcctaata caaaggacat acccgaataa tatgaccatc gggatcttca gcaaggaaag
26221 tacggccaaa aacttcggta tggggttcct gtactatttt gatctcaggg ttcttcgcc
26281 attcttcaaa gcaccgatca acatctttac cggatggcag cataatacca atttcagaaa
26341 atcgcggaat agcacgatca ggctttgctc ctccactcca aatagcaaac agtacttcac
26401 caccagcagg aatgccaca taacgggagc tggcaaatat cggttcagca ttgaaaattg
26461 ttttataaaa agcggttgaa ctctcgatat cagagacgta aacaagctga agatctgggt
26521 tgggagatc tgcttcaacg acagatttca gccttaatac gctttcaacc agatcttgcg
26581 taccttgctt aaagaaactt tcggcctgaa cagcggaat atttgcatac ggttcaaatg
26641 aaacggtcac ctgaacacga cagccatata tgttttcac tgtaataatt tgatgtcgga
26701 atgttaatat tcccatttga ggaagggttaa cctgatcggt gaattcttta ttactcttga
26761 tatttgaag atttcaacga atttcgggca ttccacttaa tatcattctg ccgtattgac
26821 ccggtttgac ctcccttcca aattgaaaat attcaagatc gacttcccat ttttccgca
26881 aatcaaaatc gacatagtgc gccagatata gggatgggtt agcgtttacc gatgtagaaa
26941 aattgagtg taacatgaca aaactctcac agttaaatgt atcacattga agtataagcg
27001 agctttttaa tacagttaga cagcccgag agtatcaatg gtgagtgaac atttctgtca
27061 gtagtcttta tttggctaac gagaaatttt ttccattgct ctccattctt tgagcaatac
27121 ttgccttgaa cgggggtaac atttggtttt aattttcaaa cgcatgattc tatctgatct
27181 gaaatgcaga aattcctcgc gtaattcaca ccattccaatc acaatgctga tattttcaaa
27241 atagcctaag gcaaatggcc atattgttct ttctgatggg atgtctttta tatccaaata
27301 agcgagggtg attttatgcc gggatattgat cgcctgacgt atctgctgaa tctcgacaac
27361 aggtgaaca gctgtcgag cagggccgat cagtaaggag cttgcctcca acatttggtt
27421 caattctgct gggatcacag ccgcaatttt gcttattgca ttatttgag ctatttttag
27481 ttgggggtct gcacgtttag ccaccaat tccaggcctc gccaacgcct ctattcccg
27541 ttgtgtaaac atgagcggtg gtaacacaaa tccaggcctc aaaaacgtatc ctattcccg
27601 ctcaccttcg ataatcgcg ccttgagcctg caacgatgca atatcccgat acagtgttct
27661 taagctgata ttcaatttct gcgccaacac ttttccctga accggaaagt gataacggcg
27721 caatatttcc atgagaaata acaaacgctg tgctctagac aattcgctcc atccattcaa
27781 gtttaaccgac tcatcaacct ctaataatac gcaactatca taatttaatt gattaaaaag
27841 atagcttttt aatgctgata aatgacctc tggtatctga ttgccctttt agattttta
27901 ttttattaat cccgtttcca aaacttactg cgtcctatcg agagaaaaat gaccatatac
27961 gattttaaacc caaatcagg cactttaacc ggcgtgttcc taatttatct gtataaacia
28021 gggatcacgc caaatcagg tgaatgccat gctgtgttcc tgccaatctt tgccgggtca
28081 ctattgagcc tatttccctc gcccacctc ttttgggtgc tgcctgtttt bcttttcatt
28141 cgctgagctc tttataatga attgggggat gtcatttctg aacataacca gaagtctcat
28201 ctgggagcta ttctgctt taccgatgt gaacagcctc atgttgccct ctacctcccc
28261 ttctgctt tcatcgcggt actggcaca acgattgggt catcacggcg cctcactatc
28321 ttgaccgaat cagatgacga ttgctttatc ttcggagctt atggattgat ctatgagcg
28381 ccgtagagaa ccttgggctg gagtatctct ttggttgctt tcatgatcat tttactctg
28441 ttcccttttg atcagcgcgt tggttaaagg catgacacca tccggctggc tgaacagtca
28501 gtgacttgct gaggcgttaa gaggcgttaa caactcgatc aacgtattgc tgaagaacat
28561 cactccaaat tattcacca catcagataa ttttaccgtt actggccaac acaacaggcc
28621 tattcacca gagcgatcat tttttttcac agcactcagg acgtatccag
28681 aatccagaca acggactcga tctgcctgat tctgcctgat tgcccgtgga
28741 catgtcgttg cagaagggcc ggcgggttac agcccatcca gattcgtgat
28801 caggttaaga ttgtcagatt tattgccact cagtaacgca tgggaacgtc
28861 gttgatgaat agagtgtcgg agcggatta gtcctgctt tcgcatgga
28921 gttatcgccc ccatgatcct cgcagcacc gcatgtgata gggtaacgca
28981 aaaatccgcg gactgcaatt gatgcaaaaa cctcttataa ctatcgcca
29041 gccacgcagg gtgaaagcca gatactgac cgtcaatatt ttaaccctgt
29101 ttgattaccc gggaaatcgc cattacacta ttttatcagc agctaaatac
29221 gtagttaaag atgccgccc acagcatcag gatacgttgg agtgaataa
29281 tatgtagtga gaaacacat tgatggatgg cgtatttttg cacttccgag
29341 gtttttgaca aaatccgggt ctattattgag tctgcccgat aatttcgaac
29401 gtttttgaca aaatccgggt ctattattgag tctgcccgat aatttcgaac
29461 gattacagcc aagaagatac gtctgtgtcc tgatgcgtaa
29521 tcattaccgt actcactggg gcagaacttc tgaattatgt tcaaaacggg
29581 ggctccacat tagattatgt ctaccgcaac gtaagggcat
29641 atactcgata agcattatct gaacagcatt ggttggcgcg
29701 catattgaaa tggtgatccg ccatgctatt ggttggcgcg
29761 catatgggtg atatcgccgc cggacacgga cgctatattc

```

Fig. 2(vi)



9/14

chr15ed2.seq

29881	agcaaagtgc	attctatctt	gttaagggac	tatagcgaaa	tcaatgttaa	tcaagggcag
29941	gcttatattg	aggagcgcga	tctgacggac	aaaattcggt	ttattatcgg	tgatgccttt
30001	aatgctgaaa	gcatctcatc	cattacgcga	gcgcccgcac	tggttattgt	atccggtctc
30061	tatgaattgt	tccctgataa	taatttactc	agaaattcgc	tacggcggtt	tgctgatggt
30121	atgacagaaa	atggttatct	ggtgtacacc	ggccaaccgt	ggcatccaca	aattgagggtc
30181	atcgcccgtg	ttctttccag	ccatcgtgac	agtcacccgt	ggatcatgcy	gcgcccgtact
30241	caaggggaaa	tggacgcatt	agtgggaagc	gccgggtttg	aaaaactgta	ccaaactgaca
30301	gataactggg	gcattttcac	tgtttcgatt	gccaaagcgtg	ttcatcgctg	atgaataaat
30361	aaatataaga	tggaaacacca	catgcactct	tctctcgata	gtcgtcggcg	cctatggctg
30421	acagggtgta	tctggctatt	gtttctggct	ccgtttttct	ttcttactta	tggccagggtc
30481	aatcagttca	cggcacaaaag	aagcgatgtc	ggcactgtga	tggtcgggtg	ggaacataac
30541	atcccttttt	ggtcatgggtc	gattatccct	tactggagta	tcgatctggt	ctacggaata
30601	tcgttattta	tctgtaccca	tcgcccgtgaa	cagtggcttc	acggctggcg	attaatgacc
30661	gcataactga	ttgcctgtgt	tggaattctta	ctgttccctc	tgaaattgta	gttctccgc
30721	cccaccacag	aaggcctatt	tggctgggtta	tttaatacaac	tgaggttatt	tgatctggcc
30781	tataatcaag	ccccttccct	gcacattatt	ctgctgtggt	tgctctggct	gcgctattca
30841	gectacgtga	gtggttactg	gcgtgggttg	ctgcacattt	ggtcagtgtc	gattgcactc
30901	tcggttctga	cgacttggca	gcacattttt	atcgatgtac	taacgggttt	tgccgttggt
30961	gtcatcctca	gttacctact	gccggtttca	taccgctggc	gctggcaacc	taatcaagat
31021	cgctatgcac	ggaagtattt	cggctattat	ctgacaggca	gcgctttggt	cgcgcttata
31081	gcgagtcgtc	tgggggggag	tttctggata	ctgctgtggc	ctgctgtatc	gttactgatg
31141	atcgactggt	gctacgcagg	attagtcagc	tccgtgtttc	aaaaacagcc	agatggccgg
31201	atgtcactgt	ctgcacgctg	gctactggcg	ccataccaac	tgaggagcatg	gctctcttat
31261	ctctgggtcc	ggcgtaaaag	cgcacctttc	aaccatataa	ctgaagggat	tattctcggc
31321	agcctgcctt	gccagcccgt	tacggcggtc	agtgtccttg	atataaccgc	tgagtggcac
31381	aggcgatcgg	atgcccgcac	agtaaatatt	gtttgccagc	cgcaaatcga	cttactgcgc
31441	ctggcacctg	aagctctaca	atcggcagtt	tgtagcgtgg	ataaactacg	ccagcaggga
31501	gatgttttcg	ttcattgtac	gcttggactg	tcacgcagtg	cgatgggtgt	agcagcatgg
31561	ctactgaaac	agcatcctga	atatgatatac	aacactgtcg	tagcaatcct	gcgtaaagcc
31621	agaccgcatg	tcacgttcag	acaaacacat	ctggatgccc	tgtctcaatg	ggcaaaaggc
31681	tacctaatac	ggggaaacata	acatgcagcc	ggaaaatctg	atcagcaaaag	tgattatcgc
31741	aacgttaaaa	agctggcgct	ttatatccac	actatctgct	ttttctatcc	tgatcgcgac
31801	tgcaatgctg	attgctgtct	tcaacactac	cgcttataac	aacattgcac	tctatgccgt
31861	actattattc	acaacgctgt	actgccataa	ctattgttgg	cgacttggc	ttgactgcca
31921	ctattttcag	atcctcaatt	catccctga	aaaaagcgcc	gagttcgatc	aaacgttatt
31981	gctgataatt	aacaagttaac	cccaatcacg	gacacagaat	gatcgcttta	acggagcctg
32041	caaaactgta	aaaaaggcta	cgatttggct	gatcctgcaa	tggaactgtt	ttttcctggt
32101	tctgcttact	ttgaaatatt	cagcctgaat	tcagctttta	tctgacaaag	tcagaacgta
32161	acgatgctac	attttctcga	ctgtactact	attaactaac	ttttagacct	gtaaacacct
32221	tattgggtga	taaaagagat	actcttacct	attctttttc	agcccgctcat	gcggagcctc
32281	tatttttttg	tttttagatta	aatgaatact	cgtaaaatta	atgggatacg	cccatttagt
32341	gcgtttattg	actcatgtct	gaaggatctt	tactctttcc	cccgttttat	cagagatata
32401	atcgctggga	ttacogtggg	tgattatgct	atccatttgg	caatggctct	ggcgatagga
32461	agcggagtgt	caccacagta	tggcctgtat	actgccgcta	tcgcccgcac	tgatcatgcc
32521	atgaccggag	gctcacgtta	tagcgtttcc	ggcccaacgg	cggtttttgt	ggtgatcctt
32581	tatcctgttt	cacagcagtt	tgggttgagc	ggcctgtcca	ttgcgacgct	gatgtcaggt
32641	gtgattttga	ttgtgatggg	attggcagct	tttgggccgg	ttatcgaata	tattcctatg
32701	tctgttacct	ttggatttac	ttccggtatt	gccatcacta	tcgctaccat	gcagggtgcaa
32761	aatttctttg	gcctgaaact	ggcacatata	ccggaaaatt	atattgataa	ggtagtgtgca
32821	ctttatcaag	ccctgccttc	attacaattg	agtatacgc	ttatcgggct	gactacgctt
32881	ctggctactga	ttttctggcc	gaaactggga	gtaaaagtta	cgggtcactt	acccgctttg
32941	atcgccggta	cagctgttat	gggcgcaatg	catctgctga	accatgatgt	cgccaccatt
33001	ggttcatcgt	ttagttacac	actggcggat	ggtacgcagg	gtcaaggcat	tccccccatt
33061	cttccccaat	ttgtcctgcc	gtggaatttg	cccgcatacac	attctctcga	tattagctgg
33121	aataccgcat	cggcattgct	gcccgcgtcg	ttttcgatgg	ccatgctggg	tgcgattgaa
33181	tcgttactgt	gtgcagtgtg	tctggatggt	atgacaggga	aaaaacacca	ttctaaccgt
33241	gagctgcttg	gccaggggtt	gggcaatatt	gccgcccctt	tctttggttg	cattaccgcc
33301	actgcggcta	tcgcccgttc	agccgccaat	gtgcgggcag	gtgcaacttc	cccgatagcc
33361	gctgtggtgc	attccctgct	ggtattatta	acattgctgg	ttttggctcc	gatgctctct
33421	tatttaccac	tggcggcaat	gtcagccatt	ctgctgattg	tcgctgggaa	tatgagcgag
33481	gcacataagg	tagtggattt	aatacgccat	gcgcctaaag	atgacattat	tgcatgctt
33541	ttgtgtctgt	cattgactgt	cctattcgat	atggctccgtc	gcgatcacta	tcggcaattgt
33601	gctggcatca	ctcctgttta	tgcgcaaaat	tgccaatatg	actcgaatca	gcacgtcatc
33661	tttaacaagc	gcggagaaaag	ggttattggt	cgtacgaatt	aacggccctt	tattctctgc
33721	tgccgcgcaa	cgtatttttg	ccgaactgag	agaaaaaagt	gctgattatc	aaaccatcat
33781	catgcagtgg	gatgccgtac	ccggttttga	cgctggcgga	ttacacgctt	ttcagggttt
33841	tgtgcgcgaa	cttggcacaag	aaaaacatat	cgctgcatgt	gatattccct	tccagccatt
33901	gaaaacgctg	gcaagagcca	aagttatgcc	gattgaagga	gagctgagtt	tttatgctac
33961	cttaaccgaag	gcactaaaag	agatggcagt	tgattacacg	cctgaggtgt	gtgcttcttc
34021	agaaaagatt	caaggctcagt	aacaacaagt	cacctctcgc	cagttatgct	ggcagagggt
34081	gactaataga	agactagcgt	aggaatttat	ttgctgggat	ccagttccgg	gaaatttttc
34141	accacgtcat	caatcgcttt	aatttgcatac	aggaatgact	ccagttttgc	caatggcaaa

Fig. 2(vii)

10/14

chr15ed2.seq

```

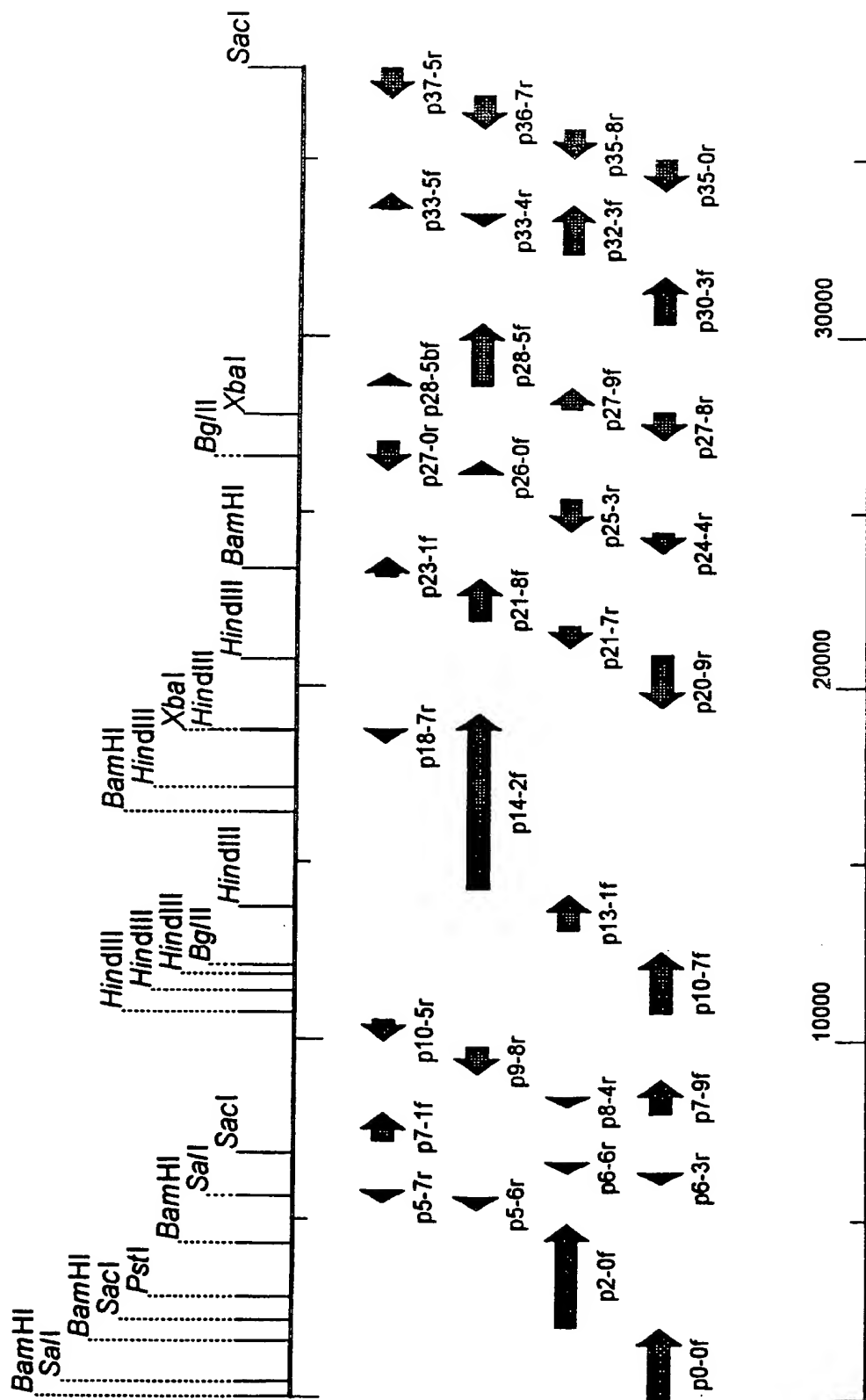
34201 gcagacgggc catcacattt ggcattttct ggatctgggt gcgcttcaag gaacagacct
34261 gcgatcccca cagccatacc cgcacgagcc agttcagcaa cctgcgcgcg acggccaccg
34321 gaagctgcac ctaatgggtc gcggcactgt aatgcatgag tgacgtcgaa aatgaccggc
34381 gcaccttggg tagcttggtg catgacaccg aagcccagca tgtcaaccac cagattgtca
34441 taaccaaagt tgctgccacg gtcacacagg atcacctggt cattgccgcg ttctttgaat
34501 ttttcaacaa tattgcccat ttgcccaggg ctaacaaact gtgggtttttt aacattaatc
34561 acagcaccgg ttttcgccat cgcttcaacc agatcgggtc ggccgggcaag gaaggctgga
34621 agctgaatca catccaccac atccgcgact ggttgagcct gtgcaggctc atgcacgtca
34681 gtgataattt tcacgccgaa gggttggttc agttcctgaa agattttcat cccctcttcc
34741 agccctggcc cagcataaga gcggatagaa gaacgggttag ccttatcaaa agacgcttta
34801 aaaacataag gaatgccag tttttgtgtc acggtgacat agtgctcaca aatccgcatg
34861 gccaaatccc gtgattcaag gacgttcatt ccaccaaaata atacaaatgg cagatcattt
34921 gcgaccttga tatcaccaat attaaccaact ttctgttgca tgcttatacc ttctcttggt
34981 aagggcaaat aaatttgctg tatcaataaa aaaataaacc taatgcagga caatcaaccg
35041 ctgtttctatg gtgttaattt gcatttttat catttctgag attgggtcct cagggcaatg
35101 ttcgacaaaa taactcaaat ccgaaacagc aatatgattg cagtcgagct gggcatagat
35161 aagcccccca tcgcggattt catagggatc atcgggatca aacgtcagca ccacttcgct
35221 ggccttaagc gccagttcca tctttttctc ttccatcaga gaaactttga tgggtgtcagt
35281 gattttgcgc acgaggctga cattatcggc ttccctgcaa tctgtttttc tcagacgaac
35341 agttgggcca atattacctt ttaaccagac atccagcgta tggctattca cgtatcccc
35401 attcaaggga ttaatgaacc aagtgggctg atcaagcaaa tcaatccgca atatcagttg
35461 gattggaaat atgacaggct gtaccgacag cccagcgccc tgagcaatat gcgtaaacac
35521 cgtacccaat gatacagggt agccttgacg tgaatggagt aagcgatcca gccacagggt
35581 atctgataga caatacaccc cattagctcc accaaaactc cattcccgat aaaaaagtgt
35641 tagcagcgaa tccaatttca ctttggaatc ggtaatggag gaaagcctct gccgggcttc
35701 ttcaaccaat gcagtttagc gctcactcac cagagtctga ggaaaatcag ggcggataat
35761 ttttgatacc agaataatc cgtcaatcag gggagtatta ttgaattcat aattaactat
35821 gggtttcatt tttattccat cgactatcg ttagcggttc attaccgcca cgtatcccc
35881 aagtcgctat ctgttgataa cccttctcta tgaataggtt tctgacaacg gttccctggt
35941 tccagccatg ttccagcaat agccatccat ttggtgacag gaaatggcgc gcctgtccca
36001 caattgcctg caaatccgcc atgcccattt gtgcagcgat caatgcagtg gctggttcaa
36061 accatgatc cccttcttgc agataggat cagcgtcatc tatatacgga ggattgctga
36121 caatcatatc aaattgttgg ttaccactgc ctgcaaaacca ctcaacttgc ggattgctga
36181 cattgtgaat ggccagtttt cgggcgtttt tttcagcatt gttgtgtgcc agcatcacgg
36241 catcagagtt gatatcgacc cctgtcacat aacaatcatt ccgctcactt gccaatgcca
36301 gtgcaatcgc ccccgcccc gtccccagat ccagaatccg ggctggagaa tcaggcaata
36361 attccaatgc cttctccacc agacattcag tatcaggcg cgggatcaac gtcgctggcg
36421 atacggcaaa cggcagtgac gttcaccaat aatataagct accggtctc
36481 cctgaatgcg gcgcaccagc aggtatcaa gctgatgcaa tttctccgat gagattagcg
36541 tttcatcgaa agcaatcaga taagtacggg aacgcccgtg cacgtatccc aacaagattt
36601 ccgcatcacg cttagggctg tcactttcag acaactgggt agccgcatgt ttagccagc
36661 attggaatt cattaatcct gctctgacag tgcagacagt tgatctgcct gatattcgat
36721 aatgatcggc tgaataagca tatccagttt gccttctatc acttcatcaa ggcggtataa
36781 cgtcagattg attcgggtgat cagtcacacg cccctgtggg aagttatagg tgcggttgcg
36841 atctgagcgg tcaccagaac ccagcaaatt tcggcggttc gaggttccca cttcttggcg
36901 cttccgcac tcagcagcac ggatacgcgc tgccaatacg gacatcgctt ttgctttggt
36961 tttgtgctgg gaacgctcat cctgacattc cactacgata cccgttggga gatgggtaat
37021 tcgaatcgca gaatcggtgg tattgacgtg ctgcccaccc gcaccggaag agcgaaacgt
37081 atctattttc aaatcacccg ggctgatgtc cggttaattca gcttctggaa tttctggcat
37141 gacagccaca gtacaggcag aagtgtgaat gcgcccctga gattccggtt ccgggtacacg
37201 ctggacacga tgaccgcctg attcaaatat caagtgaacca taacccgatg caccgaaac
37261 tttggcaatc acttctttgt agccaccatg ctcgccttcg ttggcgctta taatctctac
37321 tctccagcgg cgggcttccg catagcggt atacatgcgg aacaaatctc ccgcaaatat
37381 cgcggttcca tcgccaccgg ttccctgccc gacttcaagg aaacagttgc gctcatcatc
37441 cggatctttc ggcaacagca gtagctgtag ctgctgttcc agctcttcat tacgaatttt
37501 tgccctcctg agctcttcc cgcgcatctt cgcgatttcc acca

```

Fig. 2(viii)



11/14



**chrom5ed2.seq (37544 bps)** **Fig. 3**

12/14

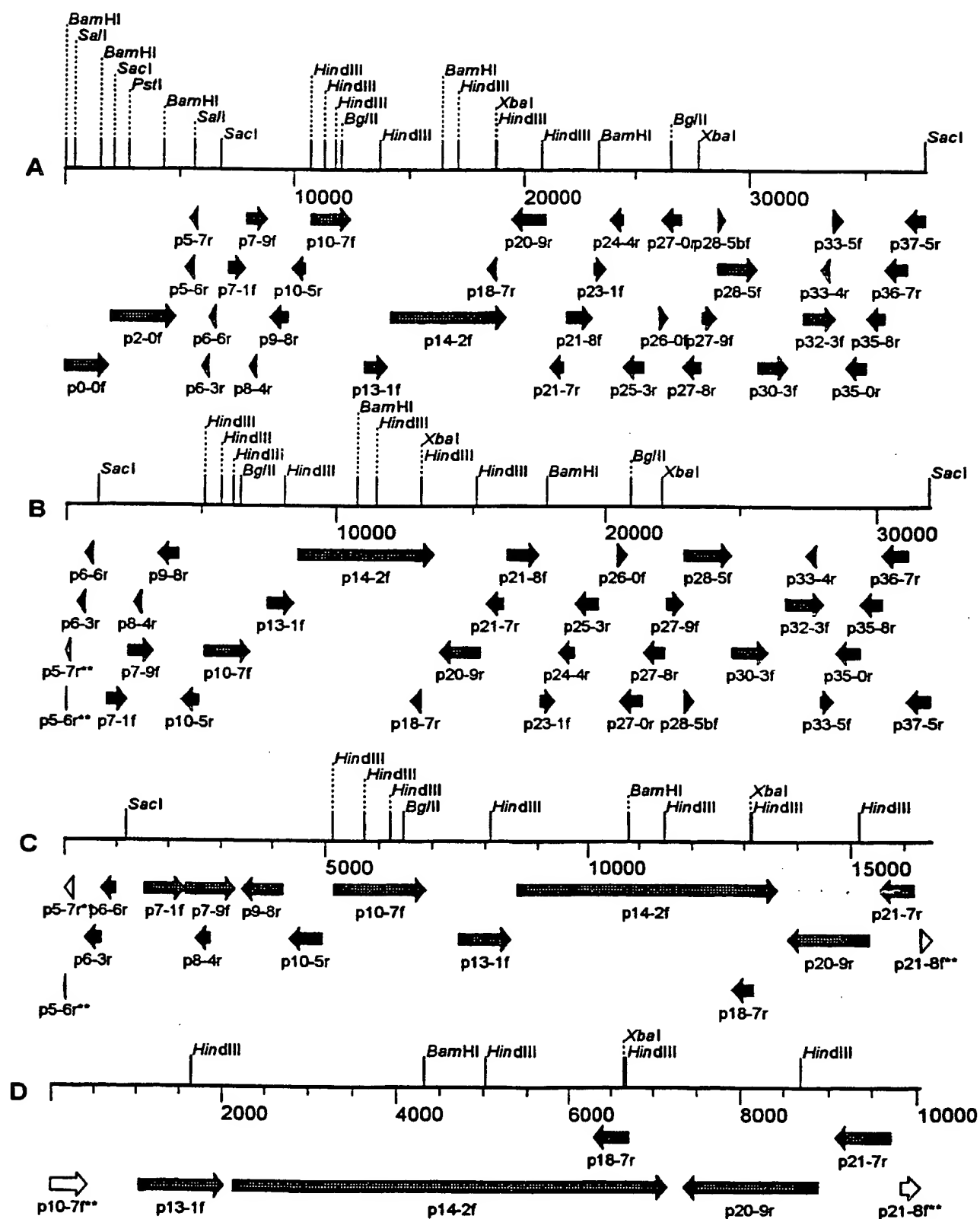


Fig. 4

13/14

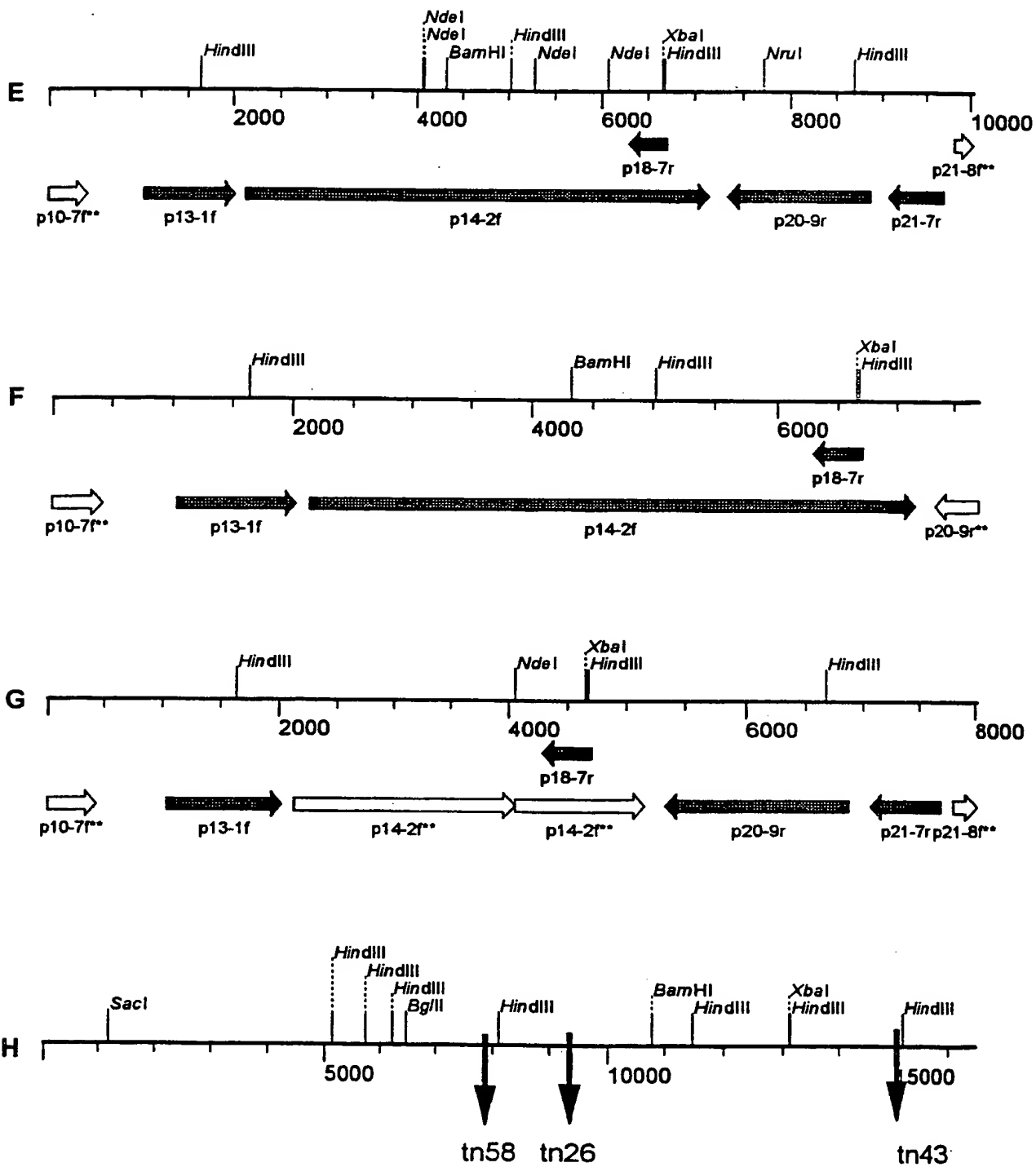


Fig. 4(cont'd)

14/14

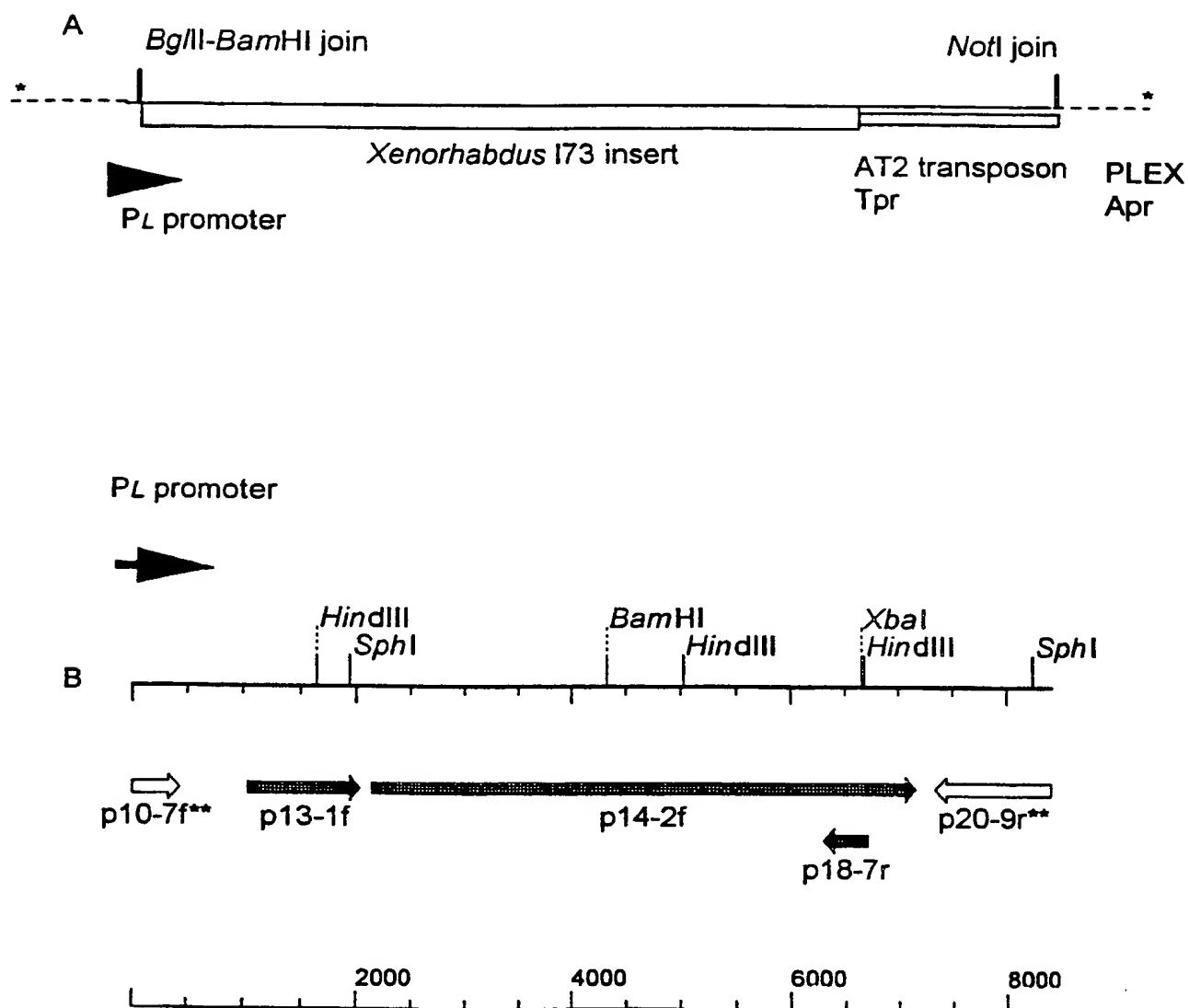


Fig. 5

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/GB 00/00219

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A01N63/00 A01N63/02 C12N15/31 C12P21/00 C07K14/24  
//(C12P21/00,C12R1:01)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>WO 99 22598 A (UNIV READING ; ELAWAD SAMI ABDULRAHAMAN (GB); HAGUE NIGEL GRAHAM MA) 14 May 1999 (1999-05-14) cited in the application page 1 - page 2 page 4, line 7 - line 14 page 5, line 2 - line 8 page 5, line 25 - line 28 page 8, line 1 - line 15; claims 1-3,9,10,14; example 5</p> <p style="text-align: center;">-/-</p>	1-9, 11, 29

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

16 May 2000

Date of mailing of the international search report

13/06/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Muellners, W

## INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/GB 00/00219

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	<p>DATABASE BIOSIS 'Online!  BIOSCIENCES INFORMATION SERVICE,  PHILADELPHIA, PA, US  AN 2000:109497,  GREWAL, PARWINDER S. (1) ET AL:  "Allelopathy: A possible mechanism of  suppression of plant-parasitic nematodes  by entomopathogenic nematodes."  retrieved from STN  XP002136524  abstract  &amp; NEMATOLOGY, (NOV., 1999) VOL. 1, NO.  7-8, PP. 735-743. ,</p>	1-9,11, 29
X,P	<p>DATABASE BIOSIS 'Online!  BIOSCIENCES INFORMATION SERVICE,  PHILADELPHIA, PA, US  AN 2000:109391,  HAN, RICHOU ET AL: "Trans - specific  nematicidal activity of Photorhabdus  luminescens."  retrieved from STN  XP002136525  abstract  &amp; NEMATOLOGY, (NOV., 1999) VOL. 1, NO.  7-8, PP. 687-693. ,</p>	1-9,11, 29
X,P	<p>CHEMICAL ABSTRACTS, vol. 132,  Columbus, Ohio, US;  abstract no. 163304,  HU, KAIJI ET AL: "Nematicidal metabolites  produced by Photorhabdus luminescens  (Enterobacteriaceae), bacterial symbiont  of entomopathogenic nematodes"  XP002136522  abstract  &amp; NEMATOLOGY (1999), 1(5), 457-469 ,</p>	1-9,11, 29
X,P	<p>WO 99 42589 A (NOVARTIS ERFINDUNGEN  VERWALTUN ;NOVARTIS AG (CH); KRAMER VANCE  CAR) 26 August 1999 (1999-08-26)  cited in the application  page 1 -page 2, paragraph 3; claims</p>	12-18
X	<p>CHEMICAL ABSTRACTS, vol. 126,  Columbus, Ohio, US;  abstract no. 234686,  GEORGIS, R. ET AL: "Novel pesticidal  substances from the entomopathogenic  nematode-bacterium complex"  XP002136523  abstract  &amp; ACS SYMP. SER. (1997),  658(PHYTOCHEMICALS FOR PEST CONTROL),  134-143 ,</p>	1-9,11, 29

## INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/GB 00/00219

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. HU ET AL.: "Mortality of Plant-Parasitic Nematodes Caused by Bacterial ( <i>Xenorhabdus</i> spp. and <i>Photorhabdus Luminescens</i> ) Culture Media" JOURNAL OF NEMATOLOGY, vol. 27, no. 4, December 1995 (1995-12), XP000905673 the whole document	1-9, 11, 29
X	WO 98 08388 A (MORGAN JAMES ALUN WYNNE ;JARRETT PAUL (GB); ELLIS DEBORAH JUNE (GB) 5 March 1998 (1998-03-05) cited in the application page 1, line 4 - line 9 page 6, line 21 -page 9, line 9 page 11, line 10 -page 12, line 19; claims 17,18,21,23-25,29	6-9, 11-30
X	PATENT ABSTRACTS OF JAPAN vol. 013, no. 286 (C-613), 29 June 1989 (1989-06-29) -& JP 01 080294 A (SUMITOMO CHEM CO LTD;OTHERS: 01), 27 March 1989 (1989-03-27) abstract; figures 2-1	12-18
A	WO 92 19739 A (MYCOGEN CORP) 12 November 1992 (1992-11-12) cited in the application	1-30
X	page 1 -page 2, line 32; claims	12-18

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 00/ 00219

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## B x II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

PCT/GB 00/00219

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9922598 A	14-05-1999	AU 9753098 A	24-05-1999
WO 9942589 A	26-08-1999	AU 3028699 A	06-09-1999
WO 9808388 A	05-03-1998	AU 4024997 A	19-03-1998
		CN 1233938 A	03-11-1999
		EP 0923295 A	23-06-1999
		ZA 9707373 A	15-02-1999
JP 01080294 A	27-03-1989	JP 2117839 C	06-12-1996
		JP 8017707 B	28-02-1996
WO 9219739 A	12-11-1992	AU 667041 B	07-03-1996
		AU 2025292 A	21-12-1992
		AU 656726 B	16-02-1995
		AU 7826491 A	12-12-1991
		BR 9205969 A	26-07-1994
		CA 2042868 A	12-12-1991
		EP 0462721 A	27-12-1991
		EP 0517367 A	09-12-1992
		HU 62928 A	28-06-1993
		JP 4229170 A	18-08-1992
		NZ 242560 A	26-01-1994
		PL 290626 A	24-02-1992
		US 5439881 A	08-08-1995
		US 5753492 A	19-05-1998
		ZA 9203168 A	27-01-1993

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>WPP81798</b>		<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. <b>PCT/GB00/00219</b>	International filing date (day/month/year) <b>24/01/2000</b>	Priority date (day/month/year) <b>22/01/1999</b>
International Patent Classification (IPC) or national classification and IPC <b>A01N63/00</b>		
Applicant <b>Horticulture Research International et al</b>		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 10 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  <b>16/08/2000</b>	Date of completion of this report  <b>15.02.2001</b>
Name and mailing address of the international preliminary examining authority:   <b>European Patent Office</b> <b>D-80298 Munich</b> <b>Tel. +49 89 2399 - 0 Tx: 523656 epmu d</b> <b>Fax: +49 89 2399 - 4465</b>	Authorized officer  <b>Donovan-Beermann, T</b>  <b>Telephone No. +49 89 2399 8213</b>



# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00219

## I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).):*

### Description, pages:

1-47 as originally filed

### Claims, No.:

1-30 as originally filed

### Drawings, sheets:

1/14-14/14 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00219

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

### 1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-30
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-30
Industrial applicability (IA)	Yes:	Claims	6-30
	No:	Claims	

2. Citations and explanations  
**see separate sheet**

## VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**see separate sheet**

## VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:  
**see separate sheet**

## VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:  
**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/00219

---

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/GB00/00219

**Ad Section V:**

The present application relates to the control of nematodes using bacteria. The bacteria are symbionts of entomopathogenic nematodes. The bacteria or agents derived from them may be used as the control agents; alternatively engineered bacteria or agents derived from them are used to control nematodes. The nematode to be controlled is normally different from that with which the bacteria is symbiotically associated. Particular bacteria used are *Xenorhabdus* strains such as *Xenorhabdus bovienii*. *Photorhabdus* is also mentioned as a typical species suitable.

WO-A-9922598 (D1, cited in the application) (see section VI below), although published between the priority date and the international filing date of the present application, is considered prior art for the present case in as far as the presently claimed priority is not valid for substantial parts of the present application. The applicant has added subject matter in filing the PCT application, which was not present in the priority filing. For that subject matter of the present application which thus has the date 24.01.00, D1 is relevant to Art.33 PCT as being prior art according to Rules 33 and 64 PCT. It may in the EP procedure become relevant to the complete present application under Art.54(3) EPC, since D1 has a filing date before the priority date of the present application.

D1 discloses biopesticide compositions for the control of insect pests and/or plant-parasitic nematodes, which use bacteria known to be symbionts of nematodes (see page 4, line 11-page 5, line 8). The bacteria species is eg. *Xenorhabdus bovienii* (see page 5, lines 25-28).

In the present application, the priority document claims the use of and compositions containing *Xenorhabdus* strain C42 but not the strains of *Xenorhabdus bovienii*. Thus the disclosure of D1 is prejudicial to novelty of the present claims in as far as they relate to this added subject matter. The presently claimed *Xenorhabdus bovienii* have the same activity as those of D1, and at present no distinction is seen. If the presently claimed strains could be proven to be novel over those of D1, it is still considered that in the light of the disclosure of D1, it would be obvious to the skilled person to seek other strains of *Xenorhabdus bovienii* which could be useful as nematocides. Thus the

subject matter of claims 9 and 10 is neither novel nor inventive (Art.33(2) and (3) PCT).

Nematology, November 1999, Vol.1(7-8), pp 735-743 (D2), is published between the priority date and the international filing date of the present application and is considered prior art for the present case in as far as the presently claimed priority is not valid for substantial parts of the present application. D2 discloses that symbiotic bacteria of entomopathogenic nematodes were able to suppress plant-parasitic nematodes. Cell-free culture filtrates from *Xenorhabdus bovienii* were effective (see Figs.3 and 4). Similar arguments apply as for D1 above, since clearly the strains of *Xenorhabdus bovienii* used in D2 are pathogenic to plant-parasitic nematodes. Thus the subject matter of claims 9 and 10 is not novel.

Nematology, November 1999, Vol.1(7-8), pp 687-693 (D3) and Nematology, 1999, Vol.1(5), pp 457-469 (D4) relate to subject matter that is fully priority-supported, and are thus of no further relevance to this application.

WO-A-9942589 (D5, cited in the application) (see section VI below), published between the priority date and the international filing date of the present application, is considered prior art for the present case in as far as the presently claimed priority is not valid for substantial parts of the present application. D5, like D1 above, may in the EP procedure become relevant to the complete present application under Art.54(3) EPC, since D5 has priority dates before the priority date of the present application.

D5 discloses nucleic acid sequences from *Photorhabdus luminescens*, which encode toxins (amino acid sequences) having insecticidal activity (see pages 1-3). Activity against plant-parasitic nematodes is not described. However, the activity described in D5 has no bearing on the fact that in the present application, claim 6 encompasses compositions containing such agents as those of D5. Indeed, on page 13 of the present application, the applicant himself states that the agents of D5, D1 etc. are useful in the present invention. The subject matter of claims 6, 7, 11-18 thus cannot be acknowledged as being novel (Art.33(2) PCT). D5 discloses recombinant vectors comprising the nucleic acid sequences and their inclusion into host cells (see paragraph bridging page 16-17). Thus the subject matter of claims 24-29 is also not novel. The probes claimed in claim 19 are parts of the nucleic acids known from D5 and

are thus also not novel. Methods for identifying or cloning known nucleic acids cannot be recognised as novel, since such methods are also used in D5 (claims 20-23).

American Chemical Society Symposium Series, 1997, Vol.658(Phytochemicals for pest control), pp 134-143 (D6) discusses the potential for using symbiotic bacteria of nematodes such as *Xenorhabdus* and/or their metabolites to control plant-parasitic nematodes (see pages 138-141). Thus the subject matter of claims 1-8 is neither novel nor inventive.

Journal of Nematology, 1995, Vol.27(4), pp 502-503 (D7) discloses the use of cell-free culture filtrates of *Xenorhabdus bovienii* effective against nematodes. As for D2, one must presume that the strains themselves were effective. Thus the subject matter of claims 1-9 is neither novel nor inventive. The subject matter of claim 10 cannot be considered to be inventive with regard to D7, since it would be obvious to the skilled person to look for alternative stains of such bacteria, with the same or similar activity against nematodes.

WO-A-9808388 (D8, cited in the application) discloses pesticidal methods and compositions derived from *Xenorhabdus* (see page 1, lines 4-9 and page 2, line 29-page 3, line 34). Nucleic acids which code for toxins in the bacteria are described. The toxins are proteins (see page 6, line 21-page 9, line 9). Methods for identifying such proteins, probes therefor, host cells transformed to express the proteins etc. are all described (see also page 11, line 10-page 12, line 19). Thus the subject matter of claims 6-8 and 11-30 is not novel.

JP-A-01080294 (D9) discloses insecticidal proteins from *Bacillus thuringiensis*, which would appear to be prejudicial to novelty of claims 12-18.

WO-A-9219739 (D10, cited in the application) discloses nematocidal toxins and genes coding for them, which are derived from *Bacillus thuringiensis* (see pages 1 and 2 and claims). This disclosure is thus prejudicial to novelty and inventive step of claims 12-18 (see also note on scope of claim 6 in section VIII).

WO-A-9717432 (D11, cited in the application but not in the International Search Report)



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/00219

discloses, like D5 and D8, insecticidal toxins from *Photorhabdus luminescens*, and forms the basis for similar objections.

For the assessment of the present claims 1-5 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Claims 1-5 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

**Ad Section VI:**

The following documents were published after the priority/filing date of the present application but have priority/filing dates prior to those of the present application. These documents may therefore be relevant to patentability of the presently claimed subject matter:

**Certain published documents (Rule 70.10)**

Application No Patent No	Publication date (day/month/year)	Filing date (day/month/year)	Priority date (valid claim) (day/month/year)
WO-A-9922598	14.05.99	30.10.98	30.10.97
"	"	"	03.11.97
WP-A-9942589	26.08.99	18.02.99	20.02.98
"	"	"	20.01.99

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/GB00/00219

**Ad Section VII:**

The certificate of deposit for *Xenorhabdus* strain X42 seems to be absent from the file.

The dependency of claim 11 does not make grammatical sense and should thus be corrected.

**Ad Section VIII:**

In claim 6, the composition is specified to contain "a species of bacterium which is a symbiont of an entomopathogenic nematode, or an engineered bacterium, or a nematode control agent derived from a natural or engineered bacterium."

Firstly, the definition of "a species of bacterium which is a symbiont of an entomopathogenic nematode" could cover a multitude of known bacteria which were however not yet known to be nematocidal. Compositions containing such bacteria cannot be accorded novelty by virtue of a new use (see section V above).

Secondly, in the present wording of the claim, the "engineered bacterium, or a nematode control agent derived from a natural or engineered bacterium" are not subject to the same definition of being derived from bacteria which are symbionts of entomopathogenic nematode, which is seen to be the central idea of the application. This subject matter is thus either vague, or non-unitary, depending on how one looks at it. In either case, it is objected to, under both Art.6 PCT and Rule 13 PCT. Claim 11 is also subject to this objection as far as the "engineered bacterium" is concerned.

The subject matter of claim 11 and therefore also the claims dependent upon it is not considered to be clear in scope as required by Art.6 PCT. The skilled person would have considerable difficulty in determining what subject matter actually falls within the scope of the present wording. The use of the phrasing "functional activity against a nematode" is too unspecific, and appear to express a desired effect rather than the concrete product.

In claim 13, similar objections apply as for claim 11. The applicant has not found such

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/GB00/00219

"degeneratively equivalent sequence" or "functional variant thereof", thus this subject matter, in any case vague, appears again to be wishful thinking on his part. The skilled person would have considerable difficulty in determining the scope of this claim.

Claim 19 is also considered to be unclear in its scope (Art.6 PCT). No further definition is put on the sequence than that it has at least 15 nucleotides, and is part of nucleic acids according to claims 12 to 18 (which are presently unclear in scope). Even if claims 12 to 18 were clearly defined, the number of shorter sequences defined by this claim would seem to be rather large, and the risk of accidental overlap with prior art also rather high. Without knowing what is encompassed by the claim, not only is it unclear, but no proper assessment of novelty can be made.

In claim 30, the "or fragment thereof" qualification to the antibody, brings the claim scope into doubt.

The patent documents listed on page 13 to which the phrase "the disclosures of which are incorporated by reference" refers, do not appear to be essential to the performance of the invention as required by Article 5 PCT. Thus this phrase should be deleted unless any of the subject matter is indeed considered to be essential; in which case it should be incorporated expressly in the description (see PCT Guidelines C-II, 4.17).